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(71) Applicant: ADVANCE TISSUE SCIENCES, INC. [US/US]; 10933 North Torrey Pines Road, La Jolla, CA 92037-1005 (US).

(72) Inventors: LEE, Ann, A.; 680 Serra Street, Stanford, CA 94305 (US). ZELTINGER, Joan; 4136 Camino Ticino, San Diego, CA 92122 (US). KLADAKIS, Stephanie, M.; 1518 Lake View Lane, Atlanta, GA 30339 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

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(57) Abstract

The present invention relates to methods for the growth of smooth muscle cells in culture for the production of tissue-engineered grafts or other implantable replacement structures. More specifically, the invention relates to the application of shear flow stress to smooth muscle cells in culture, wherein the cells align perpendicular to the direction of flow, thus more closely approximating the orientation of the cells *in vivo*. The resulting cultures and methods are useful for the production of improved vascular grafts, vessels and other implantable structures for the correction of defects or abnormal tissues in the body.

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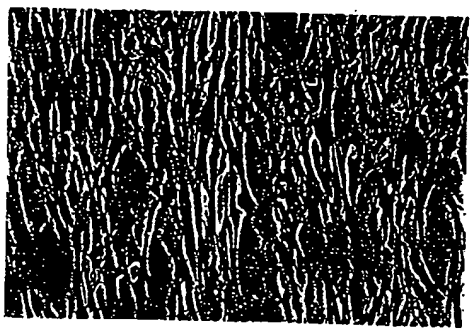

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APPLICATION OF SHEAR FLOW STRESS TO SMOOTH MUSCLE CELLS FOR THE PRODUCTION OF IMPLANTABLE STRUCTURES

The present application claims the benefit under 35 U.S.C. § 119(e) of co-pending United States provisional application Ser. No. 60/111,760, filed December 11, 1998, which is incorporated herein by reference in its entirety.

1. INTRODUCTION

The present invention relates to methods for the growth of smooth muscle cells in culture for the production of tissue-engineered grafts or other implantable replacement structures. More specifically, the invention relates to the application of shear flow stress to smooth muscle cells in culture, wherein the cells align perpendicular to the direction of flow, thus more closely approximating the orientation of the cells in vivo. The resulting cultures and methods are useful for the production of improved vascular grafts, vessels and other implantable structures for the correction of defects or abnormal tissues in the body.

2. BACKGROUND OF THE INVENTION

Vascular grafts or replacement vessels are used by vascular and cardio-thoracic surgeons to repair or replace arterial and venous blood vessels that are weakened, damaged or obstructed due to trauma or disease such as aneurysm, atherosclerosis, and diabetes mellitus. Historically, vascular grafts have been either homografts such as the patient's own saphenous vein or internal mammary artery, prosthetic grafts made of synthetic materials such as polyester (e.g., Dacron), expanded polytetraflouroethylene (ePTFE), and other composite materials, or fresh, fixed or processed biological tissue grafts (see, e.g., Vascular Surgery, Fourth Edition, Haimovici et al (eds.), Chapter 10, pp. 158-191 (Blackwell Science, Inc. 1996)).

However, synthetic grafts generally have inadequate patency rates for many uses, while the harvesting of homografts requires extensive surgery which is time-consuming, costly, and traumatic to the patient. Fixed

tissue grafts do not allow for infiltration and colonization by the host cells, which is essential to remodeling and tissue maintenance. Consequently, fixed tissue grafts degrade with time and will eventually malfunction.

Due to the inadequacies of these currently-available synthetic and biological grafts, as well as the cost and limited supply of homografts, tissue-engineered grafts have been developed for use in replacement therapy. See, e.g., United States Patent 5,792,603, which is incorporated herein by reference in its entirety, which discloses devices and methods for culturing tissue-engineered vascular grafts. Such tissue-engineered grafts more closely display the long term dimensional stability and patency of native arteries and vessels with normal physiologic functionality. See, e.g., Shinoka, et al., Mar. 1998, J. Thorac. Cardiovasc. Surg. 115(3): 536-545. See also, United States patent application Ser. No. 08/487,749, directed to three-dimensional tubular tissue-engineered cultures including blood vessel cultures, which application has recently been allowed and is also incorporated herein by reference.

In forming these tissue-engineered cultures, it is possible to grow the appropriate cells or combinations of cells corresponding to the biological structure sought to be repaired or replaced. For the repair or replacement of arteries, for example, smooth muscle cells and/or endothelial cells should be utilized since arteries in vivo are muscle tubes lined with a thin layer of endothelial cells. More specifically, blood vessels are composed of three layers: the intima , the media, and the adventitia, in order from inside to outside. See, e.g., Textbook of Histology and Color Atlas of Histology, both: 2nd printing, 1986, Finn Geneser (ed.), Lea and Febiger, Philadelphia PA. The main cellular component of the intima is the endothelial cells, the main cellular component of the media is the smooth muscle cells which produce much of the extracellular matrix of the vascular tissue, e.g., proteins such as elastin, proteoglycans, glycoproteins and collagen (see, e.g., Thie et al., 1991, Eur. J. Cell Biol. 55: 295-304). In arteries, the internal elastic lamina, which lies within the intima and exterior to the media, is a homogenous layer of fenestrated

elastin. The abundance of elastin in their walls gives arteries the ability to stretch with every contraction of the heart. Veins can also have non-homogeneous or individual fibers of elastin within their walls. The adventitia is composed of mainly fibroblasts, more ordinary vasa vasorum and some nerve tissue. The adventitia of arteries, not veins, also has an external elastic lamina that is homogeneous and fenestrated. The collagen and additional individual elastin in this layer is important for anchoring the vessel. Veins are also tubular structures lined with endothelial cells but the connective tissue layers are less well-delineated than those of arteries and the walls are thinner. Human veins also lack the internal and external elastic lamina. Thus, tissue engineering of vascular grafts can involve the culture of smooth muscle cells, endothelial cells and/or fibroblasts.

Historically, the culturing of vascular grafts, and tissue in general, has taken place in a static environment such as a Petri or culture dish. However, such static environments can lack the circulation of nutrients required for proper growth, resulting in slow and ineffective cell growth. Moreover, cells that are cultured in a dynamic environment can be exposed to conditions that more closely resemble the native in vivo environment and therefore these cultured cells are more likely to tolerate the physiological conditions to which they will be subjected once implanted in the body.

In fact, a number of studies have sought to apply various mechanical and hemodynamic forces to the growth of vascular wall cells in cell culture in vitro. For example, Benbrahim et al., 1996, J. Surg. Res. 65: 119-127, describes a vascular simulating device (VSD) in which human endothelial cells, bovine endothelial cells and bovine smooth muscle cells were cultured on coated and uncoated silicone rubber in a tubular configuration and the effects of arterial pressure, flow and strain conditions were studied. The application of mechanical stress or strain to the growth of vascular wall cells such as endothelial and/or smooth muscle cells in vitro has also been studied by Mills, et al., 1997, J. Cell Physiol. 170(3): 228-234; Takemasa et al., 1997, Exp. Cell

Res. 230: 407-410; Kanda et al., 1994, Cell Transplantation 3(6): 481-492; Kanada et al., 1993, ASAIO J. 39: M561-M565; Kanda et al., 1992, ASAIO J., 38(3): M382-M385; and Buck et al., 1983, Artherosclerosis 46: 217-223.

Shear flow stress is another type of force that has been studied for its effects on vascular wall cells in vitro. It is known, for example, that endothelial cells align parallel to the direction of shear flow stress in culture (see, e.g., Ziegler et al., 1995, Cells and Materials 5(2): 115-124; Levesque et al., 1985, J. Biomech. Eng. 107: 341-347; and Remuzzi et al., 1984, Biorheology 2: 617-630). Studies also indicate that endothelial cell alignment is dependent upon several cellular pathways such as intracellular calcium dependent pathways and microtubule reorganization (see, e.g., Malek et al., 1996, J. Cell. Sci. 109: 713-726).

Shear flow stress has been shown to affect vascular smooth muscle cells in the following ways: (1) it causes a decrease in smooth muscle cell proliferation (Ueba et al., 1997, Arterioscler. Thromb. Vasc. Biol. 17: 1512-1516; Papadaki et al., 1996, Biotech. Bioeng. 50: 555-561; and Sterpetti et al., 1992, J. Cardiovasc. Surg. 33: 619-624) and DNA synthesis (Sterpetti et al., 1992, supra); (2) it causes the release of growth factors such as PDGF, b-FGF, and TGF-β1, by the cells (Sterpetti et al., 1994, Eur. J. Vasc. Surg. 8: 138-142 and Sterpetti et al., 1993, Surg. 113: 691-699); and (3) it stimulates the release of vasoactive substances such as PGI2, PGE2, and NO (Papadaki et al., 1998, Am. J. Physiol. 274: H616-H626 and Alshihavi et al., 1996, Biochem. Biophys. Res. Comm. 224: 808-814).

The effect of shear flow stress on smooth muscle cell orientation or alignment, however, is unclear. For example, Sterpetti et al., 1992, supra, discloses that, following the application of six hours of shear flow stress to bovine smooth muscle cells in vitro at 6 dyne/cm^2, the cells assumed a spherical morphology and aligned in the direction of flow. In contrast, others explicitly state that they observed no preferential alignment of smooth muscle cells following exposure to shear flow stress in vitro. For example, Malek et al., 1994, Circ. Res. 74: 852-860 discloses that bovine aortic smooth muscle cells exhibited neither an altered cell

shape nor an alignment in the direction of flow after 24 hours of exposure to shear flow stress at 15 dynes/cm^2. Similarly, Papadaki et al., 1996, supra, found no alignment of human smooth muscle cells in the direction of flow after 24 hours at either 5 or 25 dynes/cm^2.

Other studies involve the co-culture of smooth muscle cells with endothelial cells and the application of shear flow stress to the endothelial cells. See, e.g., Nachman et al., 1998, Surg. 124(2): 353-361) and Ziegler et al., 1995, supra. Neither study analyzed the effect of shear flow stress directly on smooth muscle cells. Although in a discussion note at the end of Ziegler et al., supra, a reviewer states that, when smooth muscle cells are grown on glass or plastic, the cells orient perpendicular to the direction of flow, the authors had not carried out such experiments and there are no publications or data in the art which substantiate this comment. Moreover, while Ziegler et al., supra, and others such as Weinberg et al., 1985, J. Cell. Physiol. 122: 410-414 and Thie et al., 1991, Eur. J. Cell Biol. 55: 295-304 describe the growth of smooth muscle cells in three-dimensional collagen gels, none of these disclosures analyzes the effect of shear flow stress on the orientation or alignment of the smooth muscle cells.

It is known, however, that smooth muscle cells in vivo, e.g., in native blood vessels, are aligned circumferentially, i.e., perpendicular to the direction of blood flow through the vessel (see, e.g., Dartsch et al., 1986, Acta Anat. 125: 108-113 and Kanda et al., 1994, supra). This native orientation is believed to be necessary to provide the vessel with sufficient mechanical strength for proper biological function under physiologic pressures (see, e.g., Tranquillo et al., 1996, Biomaterials 17: 349-357; L'Heureux et al., 1993, J. Vasc. Surg. 17: 499-509).

Thus, methods for the culture of smooth muscle cells, and the tissue-engineered cultures produced therefrom, wherein the cells can be caused to align in a manner more closely resembling the orientation of native smooth muscle cells in vivo would be most useful for the production of more effective vascular grafts and other implantable replacement structures.

3. SUMMARY OF THE INVENTION

The present invention relates to the application of shear flow stress to the growth of smooth muscle cells in vitro for the production of both monolayer as well as three-dimensional tissue-engineered cultures, wherein the cells are oriented or aligned perpendicular to the direction of flow. According to a preferred embodiment, the smooth muscle cells, with or without other cells, e.g., fibroblasts and/or endothelial cells, are grown in culture under shear flow stress for the production of vascular grafts or replacement blood vessels wherein the smooth muscle cells are aligned circumferentially within the grafts, i.e., perpendicular to the direction of flow. In other embodiments, smooth muscle cells and/or fibroblasts can be grown in culture under shear flow stress for the production of skeletal muscle, ligaments, tendons or chordae tendineae, vascular linings, vascular wraps (i.e., re-enforcements) or oriented sheets of tissue for reconstructive surgery.

In accordance with the methods of the invention, the smooth muscle cells, with or without fibroblasts or other stromal cells and elements as described infra, are inoculated and grown on a substrate in either two-dimensional, e.g., monolayer culture, or in three-dimensional culture in vitro. The cells are allowed to attach to the substrate and are then subjected to shear flow stress for a time sufficient to induce a perpendicular alignment of the smooth muscle cells relative to the direction of the shear flow. Alternatively, the cells can be cultured in vitro for a desired period of time and then subjected to shear flow stress for a time sufficient to induce the perpendicular alignment of the smooth muscle cells relative to the direction of flow.

The shear flow is preferably steady and laminar and the level of shear flow stress can range from about 0.1 to about 100 dynes/cm^2; more preferably from about 0.3 to about 50 dynes/cm^2 and more preferably from about 0.3 to about 25 dynes/cm^2. The smooth muscle cells can begin to align within hours, or as long as weeks, after application of the shear flow stress, depending upon the level of the stress applied. One of skill in the art would be able to determine

empirically the appropriate level of shear flow stress to apply to obtain alignment after a desired period of time. For example, according to one embodiment, shear flow stress is applied for a duration of at least 24 hours, more preferably, for a duration of at least 48 hours at a shear flow stress level of about 20 dynes/cm^2. According to another embodiment, the shear flow stress is applied at a level of about 0.3 dynes/cm^2 for about 3 weeks.

According to a further embodiment of the invention, the smooth muscle cells are genetically modified with an exogenous gene, which gene is preferably under the control of a regulated promoter, to express a gene product beneficial for successful and/or improved transplantation/implantation, function or improved maintenance of the disease state. For example, in the case of vascular grafts, the smooth muscle cells can be genetically engineered to express anticoagulation gene products to reduce the risk of thromboembolism, atherosclerosis or occlusion, or to express anti-inflammatory gene products to reduce the risk of tissue rejection. The cells can also be genetically engineered to "knock out" expression of factors or surface antigens that promote clotting or rejection. In addition, the genetically-modified cells may produce a factor through the control of shear stress (and possibly shear stress control elements) such that the factor (locally or systemically delivered) helps to maintain or regulate other physiologic functions (e.g., prevent thrombosis or even produce insulin at constant levels).

According to another preferred embodiment, the smooth muscle cells are cultured, with or without other stromal cells such as fibroblasts, under shear flow stress on a three-dimensional framework composed of a biocompatible non-living material to form a three-dimensional living tissue construct. Upon exposure of this construct to shear flow stress, the smooth muscle cells align perpendicular to the direction of flow, resulting in a three-dimensional smooth muscle cell tissue construct, wherein the cells are aligned in a manner that more closely resembles that of native vascular vessels or structures.

In a further embodiment of the invention, the perpendicularly-aligned three-dimensional smooth muscle cell tissue construct is further seeded with endothelial cells and shear flow stress is applied to the endothelial cells to induce a parallel alignment of the endothelial cells, thereby producing a vascular graft which closely resembles native blood vessels, i.e., wherein the smooth muscle component is aligned perpendicular to the direction of shear flow and the endothelial cell component is aligned parallel to the direction of flow.

Yet another embodiment of the method of the invention comprises culturing smooth muscle cells in a monolayer culture under shear flow stress and forming the aligned cells into a tubular structure to produce an improved tubular vascular graft. According to another embodiment of this method, the perpendicularly-aligned smooth muscle cell monolayer can be further seeded with endothelial cells (either prior to or after tube formation) and subjected to shear flow stress for induction of a parallel alignment in the endothelial cells to produce an improved vascular graft containing at least one smooth muscle cell layer and an endothelial cell lining.

The methods of the invention for culturing smooth muscle cells and the tissue constructs of the invention that result therefrom are useful for the production of improved vascular grafts and/or other repair or replacement structures for implantation or transplantation in vivo. Thus, embodiments of the invention include improved tubular structures prepared in vitro for implantation in vivo, such as vascular grafts, comprising stromal cells comprising smooth muscle cells on a tubular three-dimensional scaffold composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, wherein the smooth muscle cells are aligned on the scaffold circumferentially relative to the tubular structure. This tubular structure may be further seeded with endothelial cells and subjected to shear flow stress such that the endothelial cells line the lumen of the tubular structure and align longitudinally relative to the tubular structure. Such vascular grafts can include arterial or venous grafts. These improved tubular structures can also

be genetically modified with at least one exogeneous gene as described infra.

3.1. DEFINITIONS

The following terms used herein shall have the meanings indicated:

Adherent Layer: cells attached directly to the three-dimensional support or connected indirectly by attachment to cells that are themselves attached directly to the support.

Stromal Cells: smooth muscle cells and/or fibroblasts with or without other cells and/or elements found in loose connective tissue, including but not limited to, endothelial cells, pericytes, macrophages, monocytes, plasma cells, mast cells, adipocytes, tenocytes, myofibroblasts, stromal cells of Wharton's jelly, etc.

Tissue-Specific or Parenchymal Cells: the cells which form the essential and distinctive tissue of an organ as distinguished from its supportive framework.

Three-Dimensional Framework or Scaffold: a three-dimensional support structure composed of any material and/or shape that (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. This support is inoculated with stromal cells to form a living three-dimensional stromal matrix. For example, the structure of the framework can include, but is not limited to, a mesh or a sponge, and can be shaped in the form of a tube.

Living Stromal Matrix: a three-dimensional framework which has been inoculated with stromal cells that are grown on the support. The extracellular matrix proteins elaborated by the stromal cells are deposited onto the framework, thus forming a living stromal tissue or matrix. The living stromal matrix can support the growth of tissue-specific cells later inoculated to form a three-dimensional cell culture.

Tissue-Specific Three-Dimensional Construct: a three-dimensional living stromal matrix which has been inoculated with tissue-specific or parenchymal cells and cultured. In general, the tissue-specific cells used to inoculate the

three-dimensional stromal matrix should include the "stem" cells (or "reserve" cells) for that tissue; i.e., those cells which generate new cells that will mature into the specialized cells that form the parenchyma or even the differentiated or tissue-specific matrix of the tissue.

4. BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1B. Application of shear flow stress to canine vascular smooth muscle cells, resulting in a perpendicular alignment of the cells relative to the direction of flow. Figure 1A shows an exemplary phase contrast image of the smooth muscle cells cultured in monolayer under static conditions. Figure 1B shows the phase contrast image of the smooth muscle cells exposed to 20 dynes/cm^2 of shear flow stress for 48 hours. The arrow indicates the direction of flow.

Figure 2. Alignment of vascular smooth muscle cells relative to the magnitude of applied shear flow stress. The phase contrast images depict smooth muscle cell monolayers following 48 hours of the indicated shear flow stress, i.e., ranging from no shear flow (static conditions) to 20 dynes/cm^2. The cells showed no preferential cellular alignment at 1 or 10 dynes/cm^2 when compared to static controls while the cells exposed to 15 and 20 dynes/cm^2 aligned perpendicularly to the direction of flow. The corresponding histograms measure cell orientation angle via image processing. X-axes range from -90° to +90° with respect to the direction of fluid flow. The histograms confirm that, as applied shear flow stress increases, the distribution of cell orientation angle changes from a random pattern to a distribution centered near -90° and +90°, indicating perpendicular alignment of the cells relative to the direction of flow.

Figure 3. Alignment of vascular smooth muscle cells in monolayer relative to duration and magnitude of shear flow stress. Representative histograms of cell orientation angle reveal that cells exposed to 20 dynes/cm^2 reorient to +/-90° relative to shear stress more quickly (by 24 hours) than those subjected to 15 dynes/cm^2 (by 48 hours).

Figure 4. Time course of smooth muscle cell alignment under shear flow stress represented by angular variance over time. Smooth muscle cells cultured in monolayer were exposed to 20 dynes/cm^2 of shear flow stress and began reorienting within hours of the onset of the shear stress. Image processing of time lapse video images revealed that angular variance of cell orientation angle decreases dramatically by 8 hours of experimentation, indicating an increase in cellular alignment.

Figure 5A-5B. Intracellular calcium is necessary for smooth muscle cell alignment. This figure depicts phase contrast images of canine smooth muscle cells cultured in monolayer under the following conditions: (A) under 20 dynes/cm^2 for 48 hours; and (B) smooth muscle cells treated with quin2-AM, a chelator of intracellular calcium, under 20 dynes/cm^2 for 48 hours. Treatment of the cells with quin2-AM inhibits smooth muscle cell alignment. The morphology of the qin2-AM-treated cells following shear is similar to that of statically-cultured cells.

Figure 6A-6B. Disruption of the microtubule network inhibits smooth muscle cell alignment. This figure depicts phase contrast images of canine smooth muscle cells cultured in monolayer under the following conditions: (A) under 20 dynes/cm^2 for 48 hours; and (B) smooth muscle cells treated with nocodazole, which disrupts microtubule networks, under 20 dynes/cm^2 for 48 hours. Nocodazole treatment of the cells inhibits alignment of the cells when compared with non-treated sheared controls. The nocodazole-treated cells assume a rounded morphology when compared to statically-cultured controls.

Figure 7. A diagrammatic representation of a shear flow bioreactor system that includes a growth chamber, a pump, a media reservoir, and connective tubing.

Figure 8. A diagrammatic representation of a shear flow growth chamber that contains concentric inner and outer drums at least partially submerged in liquid culture medium.

Figure 9. A diagrammatic representation of a shear flow growth chamber that contains a disk that rotates within the growth chamber.

Figure 10. A diagrammatic representation of a shear flow growth chamber that contains static parallel plates inside the growth chamber.

Figure 11A-11C. Scanning electron micrographs of smooth muscle cells in three-dimensional culture, on tubular polyurethane scaffolds with a 90% void fraction and 150-300 μm pore size, under a shear flow of 0.3 dynes/cm² for 3 weeks. Figure 11A: Hematoxylin and eosin staining of the three-dimensional culture, at a magnification of 70x, depicting smooth muscle cells growing within the scaffold. Figure 11B: Cross-section of the three-dimensional culture. Figure 11C: 500x magnification of smooth muscle cells on three-dimensional culture, demonstrating alignment of the cells perpendicular to the direction of flow.

Figure 12. Scanning electron micrographs of microvascular endothelial cells grown on tissue-engineered vascular grafts of the invention (TEVGs) comprising smooth muscle cells in three-dimensional culture on tubular polyurethane scaffolds having a 90% void fraction and 150-300 μm pore size, under a shear flow of 6 dynes/cm² for three days. The endothelial cells are aligned parallel to the direction of flow, an orientation observed in native vessels in vivo.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for culturing smooth muscle cells by applying shear flow stress to the cells. The present invention is also directed to tissue-engineered tissue constructs prepared from monolayer or three-dimensional smooth muscle cell cultures, wherein the cells are properly aligned to produce an improved vascular graft or other repair or replacement structure for implantation/transplantation.

The invention is based, in part, on the discovery that applying shear flow stress to a three-dimensional or monolayer culture of smooth muscle cells induces the cells to align perpendicularly relative to the direction of the shear flow. Since it is known that smooth muscle cells in blood vessels in vivo are found in a circumferential orientation, i.e., perpendicular to the flow of blood through the vessel,

the methods and constructs of this invention allow for the production of improved vascular grafts and other implantable structures that more closely resemble the corresponding tissues in vivo.

As used herein, the term "shear flow stress" refers to a fluid-borne force acting on cultured cells due to the relative movement between a liquid, e.g., a liquid culture medium, and the cells. The term "shear flow stress" is also referred to in the art as "fluid shear stress." Shear flow stress can be generated by moving liquid past static cells, moving cells through static liquid, or by moving the liquid and the cells simultaneously. The liquid can optionally contain small particles such as beads or microspheres to increase the viscosity of the liquid and therefore increase the level of shear (see equation (1) in Section 5.2, infra.

Shear flow stress is generally quantified in terms of dynes/cm^2 and is preferably steady and laminar. The level of shear flow stress can range from about 0.1 to about 100 dynes/cm^2; more preferably from about 0.3 to about 50 dynes/cm^2 and more preferably from about 0.3 to about 25 dynes/cm^2. The smooth muscle cells can begin to align within hours, or as long as weeks, after application of the shear flow stress, depending upon the level of the stress applied. One of skill in the art would be able to determine empirically the appropriate level of shear flow stress to apply to obtain alignment after a desired period of time. According to one embodiment, shear flow stress is applied for a duration of at least 24 hours, more preferably, for a duration of at least 48 hours at a shear flow stress level of about 20 dynes/cm^2. According to another embodiment, the shear flow stress is applied at a level of about 0.3 dynes/cm^2 for about 3 weeks.

According to the methods and constructs of the invention, smooth muscle cells (differentiated and/or undifferentiated), with or without endothelial cells, fibroblasts, and other connective tissue or stromal cells, are grown on a substrate in either two-dimensional or three-dimensional cultures in vitro and subjected to shear flow stress. As used herein, the term "substrate" refers to any surface upon which cells can attach and grow. Two-

dimensional cell culture can include the culture of cells as a monolayer or on a monolayer substrate. Subjecting such smooth muscle cell cultures to shear flow stress according to this invention induces the alignment of the smooth muscle cells in an orientation that is perpendicular to the direction of flow.

According to a preferred embodiment, smooth muscle cells are inoculated onto a biocompatible, non-living three-dimensional framework or scaffold support structure and subjected to shear flow stress. According to another preferred embodiment, smooth muscle cells and other stromal cells are grown on a three-dimensional, biocompatible, non-living framework and subjected to shear flow stress. According to one such embodiment, stromal cells comprising smooth muscle cells and fibroblasts, with or without additional stromal cells or elements, as defined in Section 3.1, supra, are inoculated onto the three-dimensional framework. Preferably, the cells are mammalian, and more preferably human cells, which cells are inoculated onto or into the three-dimensional framework and proliferate thereon to form a living stromal matrix.

The stromal cells used in this embodiment can be embryotic, fetal, up to adult in origin, and can be derived from the appropriate organs such as blood vessels, e.g., arteries veins, or umbilical cord or placental tissues. Such tissues and/or organs can be obtained by appropriate biopsy, surgical discard, organ harvest, or upon autopsy. In fact, cadaveric organs may be used to provide a generous supply of stromal cells and elements. However, the use of cells from allogeneic sources may result in problems associated with graft rejection due to immunological factors. Thus, in certain cases, it may be preferable to use undifferentiated or embryonic or fetal stromal cells, e.g., fetal fibroblasts. Sources of such fetal cells include the placenta and umbilical cord tissue. A fetal-derived stromal matrix may possess "generic" tissue properties that may be further directed by the addition of growth factors or by specific growth conditions.

In other instances, it may be preferable to use a "specific" rather than "generic" stromal support matrix, in

which case stromal cells and elements can be obtained from a particular tissue, organ, or individual. For example, where the three-dimensional culture is to be used for purposes of transplantation or implantation in vivo, it may be preferable to obtain the stromal cells and elements, e.g., smooth muscle cells and fibroblasts, from the individual who is to receive the transplant or implant. This approach might be especially advantageous where immunological rejection of the transplant and/or graft versus host disease is likely. Moreover, smooth muscle cells, fibroblasts and other stromal cells and/or elements may be derived from the same type of tissue to be cultured in the three-dimensional system. This might be advantageous when culturing tissues in which specialized stromal cells may play particular structural/functional roles, e.g., smooth muscle cells of arteries or veins.

Once inoculated onto the three-dimensional support framework, the stromal cells proliferate on the framework and elaborate growth factors, regulatory factors and extracellular matrix proteins that are deposited on the framework. The connective tissue proteins naturally secreted by the stromal cells substantially envelop the framework, forming a living stromal matrix or tissue construct. This living stromal matrix can further support the growth of tissue-specific cells inoculated onto the matrix.

As noted above, the smooth muscle cells may be inoculated onto the non-living framework with or without other stromal cells to form the living stromal matrix. Alternatively, fibroblasts with or without smooth muscle cells may be inoculated onto the framework to form the living stromal matrix and then smooth muscle cells are inoculated (as the tissue-specific cells) onto the living stromal matrix. In either case, these three-dimensional tissue constructs will sustain active proliferation of the culture for long periods of time. Because openings in the framework permit the exit of stromal cells in culture, confluent stromal cultures do not exhibit contact inhibition, and the stromal cells continue to grow, divide, and remain functionally active. Thus, the three-dimensional system described herein supports the maturation, differentiation, and segregation of cells in culture, i.e., in vitro, to form

components of adult tissues analogous to counterparts found in vivo.

Growth and regulatory factors may be added to the culture, but are not necessary since they are elaborated by the stromal tissue. The use of growth factors (for example, but not limited to, αFGF, βFGF, insulin growth factor or TGF-betas), or natural or modified blood products or other bioactive biological molecules (for example, but not limited to, hyaluronic acid or hormones), even though not absolutely necessary in the present invention, may be used to further enhance the colonization of the three-dimensional framework or scaffolding.

Because, according to the invention, it is important to re-create, in culture, the cellular microenvironment found in vivo for a particular tissue, the extent to which the living stromal matrix or tissue-specific cultures are grown prior to use of the cultures in vivo may vary depending on the type of tissue to be grown in three-dimensional tissue culture. Furthermore, the living stromal matrix may itself be used as a corrective structure by implanting it in vivo. Alternatively, the living stromal matrix may be inoculated with tissue-specific cells, e.g., smooth muscle cells and/or endothelial cells, and implanted in vivo, with or without prior culturing in vitro.

In addition, the cells grown in the system, e.g., the stromal cells of the matrix or the tissue-specific cells, may be genetically engineered to produce gene products beneficial to transplantation, e.g., anti-inflammatory factors, e.g., anti-GM-CSF, anti-TNF, anti-IL-1, anti-IL-2, etc. Alternatively, the cells may be genetically engineered to "knock out" expression of native gene products that promote inflammation, e.g., GM-CSF, TNF, IL-1, IL-2, or "knock out" expression of MHC antigens in order to lower the risk of rejection. In addition, the cells may be genetically engineered for use in gene therapy to adjust the level of gene activity in a patient to assist or improve the results of the tissue transplantation or other physiologic functions either related to vascular function, e.g., the expression of nitric oxide to improve patency and decrease thrombogenicity

or the expression of PAI-1 to decrease stenosis, or unrelated to vascular function, e.g., the expression of insulin.

In another alternative, the cells can be genetically engineered to block gene expression necessary for the transition of smooth muscle cells to proliferate, migrate and to lead to development of neointimal hyperplasia, e.g., by antisense oligodeoxynucleotide blockade of expression of cell division cycle 2 kinase and proliferating cell nuclear antigen. For a detailed discussion of the genetically-engineered cells to be used in the methods and constructs of this invention, see Section 5.4.3, infra.

Thus, according to the present invention, the three-dimensional construct described above comprising smooth muscle cells and/or fibroblasts and other stromal cells, is grown in a bioreactor under shear flow stress to produce, e.g., improved vascular grafts. For example, as depicted in Figure 7, the bioreactor includes a growth chamber in which the three-dimensional tissue construct is placed, a pump, a media reservoir, and connective tubing. Applying shear flow stress to the construct during culturing results in a vascular graft construct with smooth muscle cells oriented perpendicular to the direction of flow, which construct more closely resembles the condition of the smooth muscle cells in blood vessels in vivo and therefore, will be more likely to tolerate the physiological conditions to which it will be exposed in the human body upon implantation. In this manner, the bioreactor creates a dynamic environment in which to culture tissue-engineered vascular or other biological grafts or other implantable constructs.

Solely for ease of explanation, the detailed description of the invention is divided into the following sections: (1) establishment of the monolayer and three-dimensional tissue constructs of the invention (including the three-dimensional living stromal matrix and the tissue-specific three-dimensional tissue construct); (2) application of shear flow stress to the constructs; (3) alignment of smooth muscle cells under shear flow stress; and (4) uses of the methods and constructs of the invention.

5.1 ESTABLISHMENT OF THE MONOLAYER AND THREE-DIMENSIONAL CONSTRUCTS OF THE INVENTION

5.1.1. ISOLATION OF CELLS

According to the present invention, smooth muscle cells are used to inoculate the monolayer or three-dimensional cultures of this invention, either with or without endothelial cells, fibroblasts and other connective tissue cells such as tenocytes, pericytes, plasma cells, mast cells, adipocytes, etc. The specific cells utilized will depend on the type of graft desired. For example, for arterial or venous grafts, the inoculum can comprise medial smooth muscle cells and may further comprise adventitial or dermal fibroblasts and/or intimal endothelial cells.. The source of these inoculum cells may vary depending upon the implantable structure that is sought to be repaired or replaced. For example, in those instances where the graft or structure relates to small vessels, these inoculum cells (e.g., smooth muscle cells, fibroblasts and/or endothelial cells) may be obtained, e.g., from coronary, mammary, tibial, peroneal, femoropopliteal, dorsalis pedis, tarsal, arcuate or plantar arteries and/or veins, umbilical vessels or placenta. In those cases where the graft relates to medium vessels, the cells may be obtained from vessels such as, e.g., the carotid, subclavian or femoral arteries. In cases where large blood vessels are involved, cells may be derived from, e.g., the aortic, pulmonary, thoracic, aortoiliac or aortofemoral arteries (or veins, where appropriate). In cases where the repair or replacement structure is an internal organ, involuntary smooth muscle cells from the viscera of organs, such as the stomach, intestine, uterus or bladder, can be used. Such involuntary smooth muscle cells can also be utilized as cell sources for vessel, e.g., vascular, grafts.

The smooth muscle cells (also termed herein "SMCs"), fibroblasts and other connective tissue cells to be used in the constructs and methods of this invention can be isolated using well-established methods known in the art. As noted supra, these cells may be derived from embryonic, fetal, up

to adult sources and various organs, such as blood vessels, ligaments or tendons, which can be obtained by biopsy (where appropriate) or upon autopsy.

More specifically, SMCs and fibroblasts may be readily isolated by disaggregating an appropriate organ or tissue which is to serve as the source of the cells, using techniques known to those skilled in the art. For example, the tissue or organ can be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between neighboring cells, making it possible to disperse the tissue into a suspension of individual cells without appreciable cell breakage. Enzymatic dissociation can be accomplished by mincing the tissue and treating the minced tissue with any of a number of digestive enzymes either alone or in combination. These include but are not limited to trypsin, chymotrypsin, collagenase, elastase, and/or hyaluronidase, DNase, pronase, dispase etc. Mechanical disruption can also be accomplished by a number of methods including, but not limited to, the use of grinders, blenders, sieves, homogenizers, pressure cells, or insonators to name but a few. For a review of tissue disaggregation techniques, see Freshney, Culture of Animal Cells. A Manual of Basic Technique, 2d Ed., A. R. Liss,Inc., New York, 1987.

Once the tissue has been reduced to a suspension of individual cells, the suspension can be fractionated into subpopulations from which the SMCs or fibroblasts and/or other stromal cells and/or elements can be obtained. This also may be accomplished using standard techniques for cell separation including, but not limited to, cloning and selection of specific cell types, selective destruction of unwanted cells (negative selection), separation based upon differential cell agglutinability in the mixed population, freeze-thaw procedures, differential adherence properties of the cells in the mixed population, filtration, conventional and zonal centrifugation, centrifugal elutriation (counterstreaming centrifugation), unit gravity separation, countercurrent distribution, electrophoresis, fluorescence-activated cell sorting (FACS) or LDL uptake assay. For a review of clonal selection and cell separation

techniques, see Freshney, supra at Ch. 11 and 12, pp. 137-168; see also, Shinoka et al., supra. For example, antibodies that bind to SMC or fibroblast cell surface markers can be coated on tissue culture plates and then used to selectively bind their respective cells from a heterogeneous cell population. Similarly, SMC-specific or fibroblast-specific antibodies can be used in FACS techniques for the isolation of the SMCs or fibroblasts, respectively.

Vascular SMCs to be used in the methods and constructs of the invention can be isolated from various arteries by explant (see, e.g., Freshney, supra) or by collagenase digestion (Jaffe, 1973, J. Clin. Inv. 32: 2745-2756). More specifically, vascular SMCs are isolated by removal of the intima and adventitia to expose the media. Medial tissue is minced, explanted and incubated on tissue culture plastic in a minimum volume of nutrient medium containing 20% FCS, 95% humidity, 5% CO_2, and 37°C. The adventitia is treated similarly to release arterial fibroblasts. Cell growth is generally evident within 7-14 days. See Example Section 6.1, infra, for a detailed example of the isolation of vascular SMCs.

Alternatively, long non-branching arteries can be cannulated at either end, perfused with saline to remove excess blood and filled with a cocktail of enzymes including collagenase, elastase, Dnase, and soybean trypsin inhibitor to release SMCs into suspension. Likewise, freely dissected adventitia can be enzymatically treated to release arterial fibroblasts.

The isolation of fibroblasts may also be carried out as follows: fresh tissue samples are thoroughly washed and minced in Hanks balanced salt solution (HBSS) in order to remove serum. The minced tissue is incubated from 1-12 hours in a freshly prepared solution of a dissociating enzyme such as trypsin. After such incubation, the dissociated cells are suspended, pelleted by centrifugation and plated onto culture dishes. Fibroblasts typically attach before other cells, therefore, appropriate stromal cells can be selectively isolated and grown. The isolated fibroblasts can then be grown to confluency, lifted from the confluent culture and inoculated onto monolayer substrates or three-dimensional

framework supports (see, Naughton et al., 1987, J. Med. 18 (3 and 4): 219-250).

Endothelial cells for inoculation in the cultures of the invention can be isolated from vessels with ischemic times less than 3 hours using vessel-size specific methods. Large vessels are dissected length-wise 5 cm and flattened to expose the intimal surface (Gospodarowicz et al., 1976, Proc. Natl. Acad. Sci. USA 73(11): 4120-4124). The intimal surface is washed with calcium-free saline and a sterile cotton swab is rolled, with a little pressure, on the endothelial surface. The swab is swirled, thus releasing only endothelial cells into nutrient medium containing 10% serum and bFGF. The medium containing the cells is then plated onto a culture dish.

Smaller non-branched vessels are cannulated at either end, perfused with saline, then filled and incubated with a 0.02% collagenase solution for 10 min. The digest is removed and diluted with medium containing 10% FCS and centrifuged to pellet endothelial cells. The cells are plated onto a culture flask in Medium-199 with 10% FCS, antimicrobials, endothelial cell growth supplement and heparin (see, e.g., Sorger, 1995, In Vitro Cell Dev. Biol.-Animal 31: 671-683).

Once isolated, the cells are inoculated onto substrates for the production of the tissue cultures to which shear flow stress is then applied according to the methods of the invention.

5.1.2. MONOLAYER CULTURES

The SMCs, fibroblasts and/or endothelial cells isolated above can be inoculated onto various known substrates for the production of monolayer cultures of this invention. The substrates utilized can be made of any biocompatible material which allows cells to attach and grow thereon in a monolayer. For example, a non-porous scaffold structure made, e.g., from a biodegradable or nonbiodegradable material may be used. The cells can be also be plated onto synthetic, e.g., glass, plastic or metal substrates or non-synthetic or biologic man-made scaffolds of collagen, elastin or polymers, or composites, hybrids or combinations thereof. The substrates

may further be in the form of culture dishes, slides, flasks, plates, drums or discs.

According to one embodiment, the cells may be seeded and serially expanded in roller bottles, trypsinized, counted and then seeded overnight onto a biodegradable or bioresorbable scaffold which may be previously disinfected with 20X antibiotic/antimycotic solution or sterilized by irradiation and pre-treated with 10% BCS DMEM complete medium or 10 mg/ml fibronectin overnight. The scaffold can be seeded with, e.g., about 1 - 5 X 10^6 cells/cm^2, more preferably, about 3 X 10^6 cells/cm^2, by either direct inoculation of cells or by gentle agitation in a cell suspension, followed by static culture in a bioreactor.

In Example Section 6.2, infra, cultured SMCs (passage 6-8) were plated onto fibronectin-coated glass slides (2 $\mu g/cm^2$ fibronectin) and monolayer cultures were grown to confluence. A parallel-plate flow chamber was then used to apply shear flow stress to these monolayer cultures. Alternatively, the cells can be grown as monolayer cultures on the surface of drums, plates or discs, which are then placed in bioreactors for the application of shear flow stress to the monolayer cultures.

5.1.3. THREE-DIMENSIONAL CONSTRUCTS OF THE INVENTION

Alternatively, as noted supra, the SMCs, fibroblasts and/or endothelial cells, with or without additional stromal elements, can be inoculated onto or into a three-dimensional framework or scaffold to form a three-dimensional tissue construct for the application of shear flow stress. The parameters of the three-dimensional living stromal matrix and tissue-specific constructs of this invention, their maintenance as well as various uses of these cultures are described in co-owned United States Patent No. 4,963,489, which is incorporated herein by reference. Although the applicants are under no duty or obligation to explain the mechanism by which the invention works, a number of factors inherent in the three-dimensional culture system may contribute to its success:

(a) The three-dimensional framework provides a greater surface area for protein attachment, and consequently, for the adherence of stromal cells; and

(b) Because of the three-dimensionality of the framework, stromal cells continue to grow actively, in contrast to cells in monolayer cultures, which grow to confluence, exhibit contact inhibition, and cease to grow and divide. The elaboration of growth and regulatory factors by replicating stromal cells may be partially responsible for stimulating proliferation and regulating differentiation of cells in culture;

(c) The three-dimensional framework allows for a spatial distribution of cellular elements which is more analogous to that found in the counterpart tissue in vivo;

(d) The increase in potential volume for cell growth in the three-dimensional system may allow the establishment of localized microenvironments conducive to cellular maturation;

(e) The three-dimensional framework maximizes cell-cell interactions by allowing greater potential for movement of migratory cells, such as fibroblasts and smooth muscle cells;

(f) It has been recognized that maintenance of a differentiated cellular phenotype requires not only growth/differentiation factors but also the appropriate cellular interactions. The present invention effectively recreates the tissue microenvironment.

The three-dimensional support or framework may be of any material and/or shape that: (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. A number of different materials may be used to form the framework, including but not limited to: non-biodegradable materials, e.g., nylon (polyamides), Dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (e.g., polyvinylchloride), polycarbonate (PVC), polyurethane,

polytetrafluorethylene (PTFE; teflon), thermanox (TPX), nitrocellulose, cotton; and biodegradable materials, e.g., polyglycolic acid (PGA), collagen, collagen sponges, cat gut sutures, cellulose, gelatin, dextran, polyalkanoates, polycaprolactones and copolymers, etc. Any of these materials may be woven, braided, knitted, cast with porogens and then leached, etc., into a scaffold structure, to form the three-dimensional framework. The framework in turn can be fashioned into any shape desired as the corrective structure, e.g., tubes, ropes, filaments, sponges, etc. Certain materials, such as nylon, polystyrene, etc., are poor substrates for cellular attachment. When these materials are used as the three-dimensional framework, it is advisable to pre-treat the framework prior to inoculation of stromal cells in order to enhance the attachment of stromal cells to the support. For example, prior to inoculation with stromal cells, nylon frameworks could be treated with 0.1M acetic acid, and incubated in polylysine, FBS, and/or collagen to coat the nylon. Polystyrene could be similarly treated using sulfuric acid. Polyurethane scaffolds can be treated with fibronectin, e.g., as described in Example Section 6.4, infra.

For implantation of the three-dimensional culture in vivo, it may be preferable to use biodegradable matrices such as polyglycolic acid, collagen, collagen sponges, woven collagen, catgut suture material, gelatin, polylactic acid, or polyglycolic acid and copolymers thereof, for example. Where the cultures are to be maintained for long periods of time or cryopreserved, non-biodegradable materials such as nylon, dacron, polystyrene, polyacrylates, polyvinyls, polyurethanes, teflons, cotton, etc., may be preferred.

According to a preferred embodiment of the invention, the cells are inoculated onto or into a biodegradable or bioresorbable scaffold having within its microstructure a spiral or other concentric design which aids in the alignment of cells under shear stress in a circumferential orientation upon formation of a tubular macrostructure. The biodegradable nature of the scaffold material can also aid in the alignment of the cells because, over time, as the scaffold biodegrades, it will provide additional space for

the ingrowth of the cultured cells and allow remodeling of the culture tissue under shear flow. According to this embodiment, it is preferable that the material biodegrade within several weeks to several months.

As noted supra, SMCs, with or without other stromal cells such as fibroblasts, are inoculated onto the framework. Inoculation of the three-dimensional framework with a high concentration of stromal cells such as approximately 10^6 to $5X10^7$ cells/ml, e.g., $25X10^6$ cells/cm^3, will result in the establishment of a three-dimensional stromal tissue in shorter periods of time.

Again, where the cultured cells are to be used for transplantation or implantation in vivo, it is preferable to obtain the stromal cells from the patient's own tissues. The growth of cells in the three-dimensional stromal cell culture may be further enhanced by adding to the framework, or coating the support with proteins (e.g., collagens, elastic fibers, reticular fibers) glycoproteins, glycosaminoglycans (e.g., heparan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratin sulfate, etc.), a cellular matrix, and/or other materials.

The stromal cells may be inoculated onto the framework before or after forming the shape desired for implantation, e.g., tubes, ropes or filaments. After inoculation of the stromal cells, the three-dimensional framework should be incubated in an appropriate nutrient medium. Many commercially available media, such as RPMI 1640, Fisher's, Iscove's, McCoy's, DMEM, and the like, may be suitable for use. It is important that the three-dimensional stromal cell cultures be suspended or floated in the medium during the incubation period in order to maximize proliferative activity. In addition, the culture should be "fed" periodically to remove the spent media, depopulate released cells, and add fresh media.

During the incubation period, the stromal cells will grow linearly along and envelop the three-dimensional framework before beginning to grow into the openings of the framework. It is preferred to grow the cells to an appropriate degree which reflects the amount of stromal cells

present in the in vivo tissue prior to inoculation of the stromal matrix with tissue-specific cells (if desired).

The openings of the framework should be of an appropriate size to allow the stromal cells to stretch across, pack or fill the openings. Maintaining actively growing stromal cells which stretch across the framework enhances the production of growth factors which are elaborated by the stromal cells, and hence will support long term cultures. For example, if the openings are too small, the stromal cells may rapidly achieve confluence but be unable to easily exit from the mesh; trapped cells may exhibit contact inhibition and cease production of the appropriate factors necessary to support proliferation and maintain long term cultures. If the openings are too large, the stromal cells may be unable to stretch across the opening; this will also decrease stromal cell production of the appropriate factors necessary to support proliferation and maintain long term cultures. When using a mesh type of support, openings ranging from about 150 μm to about 220 μm will work satisfactorily. However, depending upon the three-dimensional structure and intricacy of the framework, other sizes may work equally well. In fact, any shape or structure that allows the stromal cells to stretch and continue to replicate and grow for lengthy time periods will work in accordance with the invention.

According to one embodiment of the invention as exemplified in Example Section 6.4, infra, fibronectin-treated tubular polyurethane scaffolds were used for the seeding of stromal cells in a three-dimensional culture. More specifically, polyurethane scaffolds were formed using the solvent-cast/particulate leaching method as described by Thomson et al., Polymer Scaffold Processing. In *Principles of Tissue Engineering*, RP Lanza, R. Langer and WL Chick (eds.), pp. 263-272 (RG Landes Co, Austin, TX) and United States Patent No. 5,514,378 (see Section 6.4, infra). The void fraction (VF) of the scaffold can vary, with a preferred range being from about 80% to about 95%. According to a preferred embodiment, the VF is 90%. The pore size of the scaffolds can range from 63-500 μm, with a preferred range being between 150-300 μm. As described in Section 6.4,

infra, smooth muscle cells seeded onto such polyurethane scaffolds and cultured under shear flow stress, e.g., 0.3 dynes/cm^2 for 3 weeks, aligned perpendicular to the direction of the shear flow stress, i.e., similar to the in vivo orientation of such cells in blood vessels.

Different proportions of the various types of collagen deposited on the support can also affect the growth of tissue-specific or other cells which may be later inoculated onto the stromal tissue or which may grow onto the structure in vivo. The proportions of collagen types deposited can be manipulated or enhanced by selecting stromal cells which elaborate the appropriate collagen type and inoculating such stromal cells onto the framework. For example, fibroblasts can be selected using monoclonal antibodies of an appropriate isotype or subclass that is capable of activating complement, and which define particular collagen types. These antibodies and complement can be used to select negatively the fibroblasts which express the desired collagen type. Alternatively, the stroma used to inoculate the matrix can be a mixture of cells which synthesize the appropriate collagen types desired. The distribution and origins of the five types of collagen is shown in Table I.

TABLE I

DISTRIBUTIONS AND ORIGINS OF VARIOUS TYPES OF COLLAGEN

Collagen Type	Principal Tissue Distribution	Cells of Origin
I	Loose and dense ordinary connective tissue; collagen fibers	Fibroblasts and reticular cells; smooth muscle cells
	Fibrocartilage	
	Bone	Osteoblast
	Dentin	Odontoblasts
II	Hyaline and elastic cartilage	Chondrocytes
	Vitreous body of eye	Retinal cells

III	Loose connective tissue; reticular fibers	Fibroblasts and reticular cells
	Papillary layer of dermis	
	Blood vessels	Smooth muscle cells; endothelial cells
IV	Basement membranes	Epithelial and endothelial cells
	Lens capsule of eye	Lens fibers
V	Fetal membranes; placenta	Fibroblast
	Basement membranes	
	Bone	
	Smooth muscle	Smooth muscle cells
VI	Connective Tissue	Fibroblasts
VII	Epithelial basement membranes, anchoring fibrils	Fibroblasts, keratinocytes
VIII	Cornea	Corneal fibroblasts
IX	Cartilage	
X	Hypertrophic cartilage	
XI	Cartilage	
XII	Papillary dermis	Fibroblasts
XIV, undulin	Reticular dermis	Fibroblasts
XVII	P170 bullous pemphigoid antigen	Keratinocytes

Thus, depending upon the tissue to be cultured and the collagen types desired, the appropriate stromal cell(s) may be selected to inoculate the three-dimensional framework.

Similarly, the relative amounts of collagenic and elastic fibers present in the stromal layer can be modulated by controlling the ratio of collagen-producing cells to elastin-producing cells in the initial inoculum. For

example, since the inner walls of arteries are rich in elastin, an arterial stroma should contain a high concentration of the undifferentiated smooth muscle cells which elaborate elastin.

During incubation of the three-dimensional stromal cell cultures, proliferating cells may be released from the matrix. These released cells may stick to the walls of the culture vessel where they may continue to proliferate and form a confluent monolayer. This should be prevented or minimized, for example, by removal of the released cells during feeding, or by transferring the three-dimensional stromal culture to a new culture vessel. The presence of a confluent monolayer in the vessel will "shut down" the growth of cells in the three-dimensional matrix and/or culture. Removal of the confluent monolayer or transfer of the culture to fresh media in a new vessel will restore proliferative activity of the three-dimensional culture system. Such removal or transfers should be done in any culture vessel which has a stromal monolayer exceeding 25% confluency. Alternatively, the culture system could be agitated to prevent the released cells from sticking, or instead of periodically feeding the cultures, the culture system could be set up so that fresh media continuously flows through the system. The flow rate could be adjusted to both maximize proliferation within the three-dimensional culture, and to wash out and remove cells released from the culture, so that they will not stick to the walls of the vessel and grow to confluence.

The living stromal tissue construct so formed can be used as a corrective structure in vivo. Alternatively, other cells, such as tissue-specific or parenchymal cells, may be inoculated and grown on the three-dimensional living stromal tissue prior to implantation in vivo.

For example, once the three-dimensional stromal cell culture has reached the appropriate degree of growth, additional cells such as tissue-specific cells or surface layer cells which are desired to be cultured may also be inoculated onto the living stromal tissue. Such cells inoculated onto the living stromal tissue can be incubated to allow the cells to adhere to the stromal tissue, and

implanted in vivo where continued growth can occur. Alternatively, the cells can be grown on the living stromal tissue in vitro to form a cultured counterpart of the native tissue prior to implantation in vivo. A high concentration of cells in the inoculum will advantageously result in increased proliferation in culture much sooner than will low concentrations.

The cells chosen for inoculation will depend upon the tissue to be cultured; according to the present invention, SMCs and/or endothelial cells from blood vessels may be chosen. Thus, in one embodiment, SMCs and/or endothelial cells may be added, as the tissue-specific cells, to the living stromal matrix described above.

The SMCs and/or endothelial cells used in the tissue-specific inoculum may be obtained from cell suspensions prepared by disaggregating desired tissue using standard techniques as described for obtaining stromal cells in Section 5.1.1. above. For a review of methods which may be utilized to obtain parenchymal cells from various tissues, see, Freshney, supra at Ch. 20, pp. 257-288.

As noted, growth factors and regulatory factors need not be added to the media since these types of factors are elaborated by the three-dimensional stromal cells. However, the addition of such factors, or the inoculation of other specialized cells may be used to enhance, alter or modulate proliferation and cell maturation in the cultures. The growth and activity of cells in culture can be affected by a variety of growth factors such as insulin, growth hormone, somatomedins, colony stimulating factors, erythropoietin, epidermal growth factor, hepatic erythropoietic factor (hepatopoietin), and liver-cell growth factor. Other factors which regulate proliferation and/or differentiation include prostaglandins, interleukins, and naturally-occurring chalones.

5.2 APPLICATION OF SHEAR FLOW STRESS TO THE CONSTRUCTS OF THE INVENTION

According to this invention, the monolayer or three-dimensional cultures described above are subjected to steady laminar shear flow stress. Applying such stress to the cultures causes the SMCs to align perpendicular to the direction of flow, a cellular orientation that more closely resembles the orientation of the cells in the body.

The shear flow stress may be steady and laminar and the level can range from about 0.1 to about 100 dynes/cm^2; more preferably from about 0.3 to about 50 dynes/cm^2 and more preferably from about 0.3 to about 25 dynes/cm^2. According to one embodiment, e.g., the shear flow stress is at a level of at least about 15 dynes/cm^2, more preferably at a level of at least about 20 dynes/cm^2 and is applied for a duration of at least 24 hours, more preferably, for a duration of at least 48 hours. According to another embodiment, the shear flow stress is applied at a level of about 0.3 dynes/cm^2 for a duration of about 3 weeks. One of skill in the art can readily determine by empirical methods the appropriate level of shear flow stress to be applied to obtain alignment within a desired period of time.

The shear flow stress can be applied to the cultured cells by a variety of means. For example, shear flow stress can be applied by growing a SMC monolayer on the surface of a rotating drum or rotating disc immersed in liquid growth medium (see, e.g., Figures 2 and 3). Alternatively, shear flow stress can be applied by growing a SMC monolayer on static parallel plates past which liquid growth medium is pumped (see, e.g. Figure 4). As described in Example Section 6.2, infra, SMC monolayers were subjected to shear flow stress at 20 dynes/cm^2 in a parallel-plate system. Exposure of the cells to this shear flow stress for 48 hours induced SMC alignment perpendicular to the direction of flow. Moreover, increasing the level of shear flow stress produced a significantly greater perpendicular cell alignment compared to static cultures.

Shear flow stress can also by applied to a three-dimensional SMC culture by establishing the culture in a

growth chamber through which liquid, e.g., growth medium is pumped (see, e.g., Figure 1).

The amount of shear flow stress applied to the cultures is controlled by adjusting the rate of rotation of the drum or disc, or by adjusting the liquid pumping rate. Shear flow stress using a parallel-plate chamber for monolayer culture is calculated according to the following equation (1):

shear flow stress = $\tau = 6\mu Q/bh^2$ dynes/cm^2

where: μ = viscosity of fluid (N sec/m^2);
Q = flow rate (ml/min);
b = chamber width (cm); and
h = chamber height (cm).

For the culture of three-dimensional tubes, the following equation (2) is used:

$\tau = 32\mu Q/\pi d^3$

where: d = diameter of tube (in cm)
μ = dynes x sec/cm^2
Q = cm^3/sec

An example of a bioreactor flow system that can be used in the methods of this invention is depicted in Figure 1. As depicted in Figure 7, a circulating flow system 16 is used with a growth chamber 11. The circulating flow system 16 includes a media reservoir 9, a pump 10, a growth chamber 11, and tubing 12.

Any sterilizable liquid container can be adapted for use as reservoir 9. One type of preferred reservoir is a sterile bag. Suitable sterile bags are commercially available, e.g., from Gibco/BRL. In some embodiments of the invention, an upper reservoir can be placed upstream of the bioreactor, a lower reservoir can be placed downstream of the bioreactor, and the pump can return liquid, e.g., liquid medium, from the lower reservoir to the upper reservoir.

The reservoir 9 can include a sterile filter to provide a direct source of sterile gas to the liquid in the system. Alternatively, the reservoir 9 can include a gas permeable tubing or membranes made of silicone or Teflon, e.g., to provide an indirect source of sterile gas to the system via diffusion. Preferably, one or more valves and a flow meter are included in the flow system.

The pump 10 is designed to transfer liquid from the reservoir 9 to the growth chamber 11, and return it, under sterile conditions. Typically, the pump 10 controls both the flow rate and pressure within the system. The pump 10 can be a peristaltic pump. Alternatively, an elastomeric bladder with an alternating pressure source can be used. Varying the external pressure causes the bladder to inflate and deflate. A pair of check valves can be used to achieve unidirectional movement of sterile fluid in the system. The connective tubing 12 for circulating the sterile liquid within the system can be stainless steel pipe or durable medical-grade plastic tubing. Alternatively, the tubing 12 can be a gas-permeable material such as silicone.

The flow system of Figure 7 can be used for culturing either monolayer or three-dimensional cell cultures. Thus, a three-dimensional culture can be placed in the growth chamber and shear flow stress applied by the movement of the liquid pumped through the growth chamber. Preferably, the three-dimensional framework containing the attached cultured cells is static.

Alternative shear flow systems that can be used include those depicted in Figures 8-10. The system depicted in Figure 8 includes a growth chamber 3 that contains concentric drums 1 and 2. The surface of either drum or both can serve as a substrate for the attachment of cells according to this invention. For example, SMCs can be inoculated onto the drum surface(s) to produce a SMC monolayer to which shear flow stress can then be applied. One of the drums can remain stationary while the other drum rotates. Alternatively, both drums can rotate. In either arrangement, the drums 1,2 are at least partially submerged in liquid, e.g., growth medium, 15. The relative movement between the drum-anchored cells and the liquid 15 generates shear flow stress.

The amount of flow stress can be adjusted by adjusting the drum rotation speed according to equation (1) above. The distance between drums 1, 2 is parameter h in equation (1). Preferably, drum rotation speed is selected to achieve the shear flow stress ranges listed supra. Preferably, the drum is rotated by means of a variable speed electric motor. The

optimal distance between the two drums depends on various factors, including drum size and the material from which the drums are made. Determination of the optimal drum configuration is within the ordinary skill in the art. Because shear flow stress is generated by the drum's rotation, a continuous flow arrangement involving a liquid reservoir and pump is optional.

The shear flow system of Figure 9 contains a growth chamber 5 that includes a rotating disc 4. The disc 4 is immersed in liquid 15, e.g., culture medium, wherein the disc serves as a substrate for the attachment of the cells. For example, SMCs can be inoculated onto the disc surface to produce a SMC monolayer to which shear flow stress can then be applied. The amount of flow stress applied to the cells can be adjusted by adjusting the disc rotation speed according to equation (1), supra. Optionally, a multiplicity of discs can be placed on a single rotating shaft to increase the total surface area available to support cell growth. Typically, the drum is rotated by means of a variable speed electric motor. Because shear flow stress is generated by the drum's rotation, a continuous flow arrangement involving a liquid reservoir and pump is optional.

The parallel-plate flow system depicted in Figure 10 contains a shear flow growth chamber 8 that includes two parallel, static plates or walls 6 and 7 inside the growth chamber 8. A single pair of static plates 6, 7 can be used or more than one pair of plates can be used in the same growth chamber 8. Because the plates 6, 7 are static, shear flow stress is generated solely by the movement of liquid 15, e.g., culture medium, pumped through the chamber 8. The liquid is pumped past the parallel plates to create the desired shear flow stress. As noted supra, the shear flow stress may range from about 1 to 100 dynes/cm^2, or more preferably, the shear stress may be at a level from at least about 15-20 dynes/cm^2. Shear flow stress is adjusted by adjusting the liquid 15 flow rate in accordance with equation (1), supra. The distance 13 between the static plates 6, 7 is parameter h of equation (1). The static plates are

preferably parallel to each other and at right angles to the prevailing flow of liquid 15.

The advantage of increasing the number of plates 6, 7 is the increase in total surface area obtained on which the cells can form a monolayer. The potential disadvantage of multiple plates is that, as the number of plates increases, it may become relatively more difficult to maintain an even level of shear flow stress on the cells throughout the chamber 8. One way of maintaining an even level of shear flow stress is to disperse the entering liquid 15a over a wide area on one wall of the chamber 8 while collecting the exiting liquid 15b from a similarly wide area on the opposite wall of the chamber.

Optionally, the shear flow stress apparatus utilized should be able to modulate or regulate medium flow rate, pH and/or temperature and may have image capture/processing means for evaluating alignment. The drums, discs and plates described above should be made of a bio-compatible material, e.g., polystyrene, polycarbonate or stainless steel. The substrates may also be made of non-porous biodegradable materials such as scaffolds as long as these substrates confine growth to a monolayer culture. Selection of a suitable material for the walls of the growth chamber and the substrate is within the ordinary skill in the art.

5.3 ALIGNMENT OF SMCs UNDER SHEAR FLOW STRESS

Applying shear flow stress as described above to the SMC cultures described herein alters the alignment of the cells relative to the direction of the shear flow stress, causing the SMCs to align perpendicular to the direction of flow. See, e.g., Example Section 6.2, 6.3, and 6.4, infra. As shown in Figure 1, the alignment of canine vascular SMCs grown in static cultures is essentially random. In contrast, the exposure of the cultured cells to 20 dynes/cm^2 of shear flow stress for 48 hours induced the cells to align perpendicular to the direction of flow. The perpendicular alignment was dependent upon the magnitude (Figure 2) and the duration (Figure 3) of the shear flow stress exposure. The perpendicularly-aligned SMC cultures of this invention

possess a cellular orientation more closely resembling the orientation of the cells in vivo and therefore allow for the production of improved SMC tissue-engineered constructs for implantation in vivo.

Alignment of the SMCs may be visualized using any method known to those of skill in the art including, but not limited to, visually or with the aid of a microscope or other optical imaging apparatus such as phase contrast or fluorescent imaging. Cells may be stained or immunostained to assist in visualization.

5.4 USES OF THE METHODS AND CONSTRUCTS OF THE INVENTION

5.4.1. FORMATION OF TUBULAR BIOLOGICAL TISSUES

The three-dimensional cultures and constructs of this invention grown under shear flow stress can be used to construct single or multi-layered tubular tissues in vitro. These tubular structures having smooth muscle tissue aligned in a circumferential orientation (i.e., perpendicular to the direction of flow) with or without endothelial cells oriented longitudinally, i.e., parallel to the direction of flow, will more closely resemble tubular tissues and organs in the body, including, but not limited to, blood vessels, ligaments, and tendons. These structures can therefore be used as improved implantable grafts and replacement structures for correcting damaged tissues in the body.

Tubular structures such as blood vessels are composed of layers of stromal tissue with an interior lining of endothelium. Their connective tissues contain layers of smooth muscle with varying degrees of elastic fibers, both of which are especially prominent in arterial blood vessels. In addition, such structures often contain fibroblasts and varying amounts of collagen. By applying shear flow stress to cells which comprise the various components of such vessels, e.g., SMCs, endothelial cells, fibroblast, etc., the methods of this invention provide three-dimensional implantable tissue constructs or structures with components that more closely reflect the tissue components found in vivo and therefore these implantable structures can attain the

special structural and functional properties required for proper physiological functioning in vivo. They can thus serve as improved replacements for damaged or diseased tubular tissues in a living body.

5.4.1a. SINGLE-LAYER TUBULAR STRUCTURES

The following subsections describe the use of various scaffold frameworks for the growth of cells under shear flow stress for the preparation of tubular structures that can be implanted into the body.

(A) FLAT MESH STARTING MATERIAL

A scaffold can be cut into a rectangular strip of which the width is approximately equal to the inner circumference of the tubular organ into which it will ultimately be inserted. The SMCs and/or other stromal cells can be inoculated onto this scaffold and incubated under shear flow stress in liquid media in accordance with this invention. At the appropriate stage of confluence, the scaffold can be rolled up into a tube by joining the long edges together to form an improved properly aligned tubular implantable tissue construct. The seam can be closed by suturing the two edges together using fibers of a suitable material of an appropriate diameter.

(B) TUBULAR SCAFFOLD STARTING MATERIAL

Alternatively, a scaffold can be formed initially as a tube, inoculated with SMCs and/or other stromal cells, and grown in media under shear flow stress in accordance with this invention. In order to prevent cells from occluding the lumen, one of the open ends of the tubular scaffold can be affixed to a nozzle and the liquid media can be forced through this nozzle from a source chamber connected to the incubation chamber to create a current through the interior of the tubular structure. The other open end can be affixed to an outflow aperture which leads into a collection chamber, from which the media can be recirculated through the source chamber. The tube can be detached from the nozzle and outflow aperture when incubation is complete. This method is

described by Ballermann, B.J., et al., Int. Application No. WO 94/25584 (see also, United States Patent No. 5,792,603). The improved properly-aligned tubular structure produced in this way can be implanted in vivo.

(C) MULTI-LAYER TUBULAR STRUCTURES

In another embodiment, at least two three-dimensional cultures grown under shear flow stress can be combined into a multi-layered tubular structure using a variety of methods. For example, a multi-layer flat scaffold can be formed by laying one flat rectangular culture atop another and suturing the two together or annealing the two together using a light-activated substance. This two-layer sheet can then be rolled up to form a tubular structure, as described above for a single culture supra, i.e., by joining together the long edges, and securing with sutures. Alternatively, one tubular scaffold that is to serve as the inner layer can be inoculated and incubated under shear flow stress and a second scaffold can be grown, also under shear flow stress, as a flat, rectangular strip with a width slightly larger than the outer circumference of the tubular scaffold. After appropriate growth is attained, the rectangular scaffold can be wrapped around the outside of the tubular scaffold. Closing the seam of the outer strip and securing it to the inner tube can be accomplished in a single suturing or annealing step, resulting in a multi-layered tubular implantable structure.

Alternatively, two tubular meshes of slightly differing diameters can be grown separately under shear flow stress. The culture with the smaller diameter can be inserted inside the larger one, and secured with sutures. This method would not be practical for very narrow tubes.

For each of these methods, more layers can be added by reapplying the method to the double-layered tube. In addition, any suitable method can be employed to shape the three-dimensional cultures to take on the conformation of the natural organ or tissue to be simulated.

(D) ARTERIAL AND VENOUS TUBULAR STRUCTURES

Preferred embodiments of the multi-layered tubular structure of this invention include arterial tubular constructs and venous tubular constructs. As noted above, blood vessels are composed of three layers: the intima, the media, and the adventitia, in order from inside to outside. The main cellular component of the intima is the endothelial cells, the main cellular component of the media is the smooth muscle cells which produce much of the extracellular matrix of the vascular tissue, e.g., proteins such as elastin. In arteries, the internal elastic lamina, which lies within the intima and exterior to the media, is a homogenous layer of fenestrated elastin. The abundance of elastin in their walls gives arteries the ability to stretch with every contraction of the heart. Veins can also have non-homogeneous or individual fibers of elastin within their walls. The adventitia is composed of mainly fibroblasts, more ordinary vasa vasorum and some nerve tissue. The adventitia of arteries, not veins, also has an external elastic lamina that is homogeneous and fenestrated. The collagen and additional individual elastin in this layer is important for anchoring the vessel. Veins are also tubular structures lined with endothelial cells but the connective tissue layers are less delineated than those of arteries and the walls are thinner. Human veins also lack the internal and external elastic lamina.

There is a compositional and functional difference between all of the layers of a blood vessel and consequently, it may be advantageous in accordance with the invention to grow the different layers on separate scaffolds. Whether the intima and media are grown on separate scaffolds, or combined in one, depends on how distinct these layers are in the particular artery into which the three-dimensional culture is to be implanted.

For example, according to the invention, fibroblasts can be isolated from the adventitia of a patient's artery and used to inoculate a three-dimensional framework, as described in Section 5.1.3., supra, and grown under shear flow stress in accordance with this invention. Cells can also be isolated from tissue rich in elastin-producing

undifferentiated smooth muscle cells, also containing some fibroblasts, e.g., from the intima and media of the same artery, and used to inoculate either a separate scaffold or the same scaffold (i.e., as tissue-specific cells on top of the living stromal matrix described in Section 5.1, supra). In either case, the two scaffolds or the single three-dimensional culture is grown under shear flow stress. Once the SMCs have proliferated to the appropriate extent and aligned perpendicular to the direction of flow, the scaffolds can be combined using one of the methods described above to form a tubular structure (if separate scaffolds are utilized). In this manner, an improved arterial graft can be produced having connective tissue layers similar to those found in arteries in vivo.

In order to produce an arterial graft that is even more similar to arteries found in vivo, endothelial cells can be isolated from the same patient (or other sources as described supra) and seeded on top of a perpendicularly-aligned SMC culture (e.g., as described above). For example, the endothelial cells can be seeded as tissue-specific cells onto a living stromal matrix containing SMCs, as described in Section 5.1.3., supra, which matrix has already been subjected to shear flow stress and has its SMCs aligned perpendicular to the direction of flow. After inoculation of the endothelial cells onto the matrix, the culture is again grown under shear flow stress, thus causing the alignment of the endothelial cells parallel to the direction of flow. The scaffold can then be formed into a tubular structure as described above, forming an implantable graft that very closely resembles arterial vessels in vivo, i.e., which contain an endothelial lining with cells oriented parallel to the direction of flow and underlying SMC layers with cells oriented perpendicular to the direction of flow (see also, Example Section 6.4, infra).

If a fully functional replacement with all the various layers of tissue is not required, a simple homogeneous three-dimensional elastin-rich, e.g., SMC-containing stromal culture grown under shear flow stress can be used. Additional layers of this homogeneous stromal matrix can be

combined to provide the appropriate thickness for such a prosthesis.

The layers of the connective tissue comprising the walls of veins are less delineated than those of arteries. Consequently, a single three-dimensional culture can be grown, for example, from a single inoculum of cells. These cells consisting mostly of fibroblasts with some smooth muscle cells, can be isolated from the walls of a vein of the patient and grown under shear flow stress as described herein to produce a culture with SMCs aligned perpendicular to the direction of flow, which culture can be formed into a tubular structure as described above. Alternatively, when the appropriate degree of confluence is reached in the stromal culture, endothelial cells, isolated from the same patient, for example, can be seeded on top of the stromal layer and grown to confluence under shear flow stress. The culture can then also be formed into a tubular structure and will comprise a venous structure having an internal endothelial layer oriented parallel to the direction of flow and one or more interior SMC layers oriented perpendicular to the direction of flow. As noted, these preferred embodiments most closely reflect the native condition of blood vessels and are therefore most likely to withstand the mechanical and hemodynamic stresses which will be placed upon them when they are implanted in vivo.

5.4.2. IMPLANTATION/TRANSPLANTATION

The tissue-engineered constructs described herein can be implanted in vivo to correct defects or replace surgically removed or damaged tissues. Implantation can be carried out with either the monolayer cultures or the three-dimensional cultures described herein. The constructs are preferably implanted to repair or replace structures containing smooth muscle cells and SMC cell layers such as blood vessels, e.g., small, medium or large vessels. Preferred implantable structures include tubular arterial and venous grafts.

In addition, the constructs are useful in the repair and replacement of involuntary smooth muscle viscera such as is found in the stomach, intestine, uterus, or bladder. Also, the constructs can be used for the repair or replacment of

ligaments, tendons and chordae tendineae (tendonous-like chords of the tricuspid and mitral valves of the heart). In the case of ligaments or tendons (which are comprised of fibroblasts and collagen fibers), stromal monolayer cultures or the three-dimensional stromal living matrix or three-dimensional SMC cultures as described herein can be used.

5.4.3 GENETIC ENGINEERING AND GENE THERAPY

The monolayer and three-dimensional constructs grown under shear flow stress as described herein may afford a vehicle for introducing genes and gene products in vivo to assist or improve the results of the implantation/transplantation and/or for use in gene therapies. According to a preferred embodiment, the three-dimensional constructs are used. For example, for vascular grafts, the stromal or tissue-specific cells of the constructs, e.g., SMCs, endothelial cells or fibroblasts, can be genetically engineered to express anticoagulation gene products to reduce the risk of thromboembolism, or anti-inflammatory gene products to reduce the risk of failure due to inflammatory reactions. In this regard, the cells can be genetically engineered to express TPA, streptokinase or urokinase to reduce the risk of, e.g., clotting. For vascular or other types of tissue grafts, the cells could also or alternatively be engineered to express anti-inflammatory gene products, for example, peptides or polypeptides corresponding to the idiotype of neutralizing antibodies for TNF, IL-2, or other inflammatory cytokines. Alternatively, the gene encoding the human complement regulatory protein, which prevents rejection of the graft by the host, may be inserted into human fibroblasts. McCurry et al., 1995, Nature Medicine 1: 423-427. Preferably, the cells are engineered to express such gene products transiently and/or under inducible control during the post-operative recovery period, or as a chimeric fusion protein anchored to the cells, for example, a chimeric molecule composed of an intracellular and/or transmembrane domain of a receptor or receptor-like molecule, fused to the gene product as the extracellular domain.

In another embodiment, the stromal or tissue-specific cells of the constructs can be genetically engineered to express a gene for which a patient is deficient, or which would exert a therapeutic effect, e.g., HDL, apolipoprotein E, etc. The genes of interest engineered into the cells need to be related to the disease being treated. For example, for vascular disease, the cells can be engineered to express gene products that are carried by the blood, e.g., cerebredase, adenosine deaminase, α-1-antitrypsin. In a particular embodiment, a genetically engineered vascular graft culture implanted to replace a section of a vein or artery can be used to deliver gene products such as α-1-antitrypsin to the lungs; in such an approach, constitutive expression of the gene product is preferred. According to another embodiment, genetically engineered cells that express wound healing factors may be incorporated into the living stromal cultures used to make tendons and ligaments to enhance wound healing at the site of implantation.

In addition, genes that prevent or ameliorate symptoms of various types of vascular diseases may be underexpressed or down regulated under disease conditions. Specifically, expression of genes involved in preventing the following pathological conditions may be down-regulated, for example: thrombus formation, inflammatory reactions, and fibrosis and calcification of the valves. Thus, expression of the gene encoding an integrin antagonist can be engineered into the cells to aid in the treatment of abnormal cell migration. Similarly, the cells can be engineered to contain the genes for, e.g., IIb/IIIa blockers for the treatment of thrombosis. See, e.g., Nikol et al., 1996, Atherosclerosis 123: 17-31.

Alternatively, the activity of gene products may be diminished, leading to the manifestations of some or all of the above pathological conditions and eventual development of symptoms of valvular disease. Thus, the level of gene activity may be increased by either increasing the level of gene product present or by increasing the level of the active gene product which is present in the monolayer or three-dimensional culture system. The culture which expresses the active target gene product can then be implanted into the valvular disease patient who is deficient for that product.

"Target gene," as used herein, refers to a gene involved in diseases sought to be treated such as, but not limited to, vascular disease, in a manner by which modulation of the level of target gene expression or of target gene product activity may act to ameliorate symptoms of the disease.

In addition, the stromal or tissue-specific cells used in the three-dimensional culture system of the invention may be genetically engineered to "knock out" expression of factors or surface antigens that promote clotting or rejection at the implant site. Negative modulatory techniques for the reduction of target gene expression levels or target gene product activity levels are discussed below. "Negative modulation", as used herein, refers to a reduction in the level and/or activity of target gene product relative to the level and/or activity of the target gene product in the absence of the modulatory treatment. The expression of a gene native to, e.g., the stromal cells can be reduced or knocked out using a number of techniques, for example, expression may be inhibited by inactivating the gene completely (commonly termed "knockout") using the homologous recombination technique. Usually, an exon encoding an important region of the protein (or an exon 5' to that region) is interrupted by a positive selectable marker (for example *neo*), preventing the production of normal mRNA from the target gene and resulting in inactivation of the gene. A gene may also be inactivated by creating a deletion in part of a gene, or by deleting the entire gene. By using a construct with two regions of homology to the target gene that are far apart in the genome, the sequences intervening the two regions can be deleted. Mombaerts, P., et al., 1991, Proc. Nat. Acad. Sci. U.S.A. 88:3084-3087.

Antisense and ribozyme molecules which inhibit expression of the target gene can also be used in accordance with the invention to reduce the level of target gene activity. For example, antisense RNA molecules which inhibit the expression of major histocompatibility gene complexes (HLA) have been shown to be most versatile with respect to immune responses. Still further, triple helix molecules can be utilized in reducing the level of target gene activity. These techniques are described in detail by L.G. Davis, et

al., eds, Basic Methods in Molecular Biology, 2nd ed., Appleton & Lange, Norwalk, Conn. 1994.

In another alternative, the cells of the constructs can be genetically engineered to block gene expression necessary for the transition of smooth muscle cells to proliferate, migrate and to lead to development of neointimal hyperplasia, e.g., by antisense oligodeoxynucleotide blockade of expression of cell division cycle 2 kinase and proliferating cell nuclear antigen. Mann, M.J., et al., 1995, Proc. Natl. Acad. Sci. USA 92:4502-4506.

Using any of the foregoing techniques, the expression of fibrinogen, von Willebrands factor, factor V or any cell surface molecule that binds to the platelet α2Bβ-3 receptor can be knocked out in the stromal cells to reduce the risk of clot formation in the vascular or other types of biological tissue grafts. Likewise, the expression of MHC class II molecules can be knocked out in order to reduce the risk of rejection of the graft.

Methods that may be useful to genetically engineer the cells of the invention are well-known in the art and are further detailed in United States Patents 4,963,489 and 5,785,964, the disclosures of which are incorporated herein by reference. For example, a recombinant DNA construct containing an exogenous nucleic acid, e.g., encoding a gene product of interest, may be constructed and used to transform or transfect the stromal or tissue-specific cells described herein. The transformed or transfected cells that carry the exogenous nucleic acid, and that are capable of expressing said nucleic acid in the form of the gene product, are selected and clonally expanded in the three-dimensional culture system of this invention.

Methods for preparing DNA constructs containing the gene of interest, for transforming or transfecting cells, and for selecting cells carrying and expressing the gene of interest are well-known in the art. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, NY; and Sambrook et al., 1989, Molecular

Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

For example, the cells of this invention can be transformed with the DNA construct, i.e., controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.) and a selectable marker. Following the introduction of the foreign or exogenous DNA, engineered cells may be allowed to grow in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which, in turn, can be cloned and expanded into cell lines. This method can advantageously be used to engineer cell lines which express the gene protein product.

The three-dimensional cultures containing such genetically engineered cells, e.g., either mixtures of cells each expressing a different desired gene product or a cell engineered to express several specific genes, are implanted into the patient to allow for the amelioration of the symptoms of diseases such as, but not limited to, vascular disease. The gene expression may be under the control of a non-inducible (i.e., constitutive) or inducible promoter. The level of gene expression and the type of gene regulated can be controlled depending upon the treatment modality being followed for an individual patient.

A variety of methods may be used to obtain the constitutive or transient expression of gene products engineered into the tissue construct cells. For example, the transkaryotic implantation technique described by Seldon, R.F., et al., 1987, Science 236:714-718 can be used. "Transkaryotic", as used herein, suggests that the nuclei of the implanted cells have been altered by the addition of DNA sequences by stable or transient transfection. The cells can be engineered using any of the variety of vectors including, but not limited to, integrating viral vectors, e.g., retrovirus vector or adeno-associated viral vectors, or non-integrating replicating vectors, e.g., papilloma virus vectors, SV40 vectors, adenoviral vectors; or replication-

defective viral vectors. Where transient expression is desired, non-integrating vectors and replication defective vectors may be preferred, since either inducible or constitutive promoters can be used in these systems to control expression of the gene of interest. Alternatively, integrating vectors can be used to obtain transient expression, provided the gene of interest is controlled by an inducible promoter.

Preferably, the expression control elements used should allow for the regulated expression of the gene so that the product is synthesized only when needed in vivo. The promoter chosen would depend in part upon the type of tissue and cells cultured. In the case of vascular grafts, SMCs, endothelial cells or other stromal cells can be genetically engineered to produce a gene product under shear stress by utilizing a shear stress promoter or other shear stress control elements, such that the gene product (locally or systemically delivered) helps to maintain or regulate vascular or other physiologic functions, e.g., prevent thrombosis or even produce insulin at constant levels. For example, vascular grafts having a constitutive expression of insulin under shear flow stress can be used to facilitate liver transplants or in the treatment of insulin-related disease, e.g., diabetes mellitus. In addition, the cells can be genetically engineered to produce the growth factor, VEGF, under the control of a shear stress responsive promoter. One such promoter may be derived from the gene encoding thrombomodulin, a protein that is transiently upregulated upon the application of shear stress.

Although shear stress responsive promoters are favored, any promoter may be used to drive the expression of the inserted gene. For example, viral promoters include but are not limited to the CMV promoter/enhancer, SV 40, papillomavirus, Epstein-Barr virus, elastin gene promoter and β-globin. If transient expression is desired, such constitutive promoters are preferably used in a non-integrating and/or replication-defective vector. Alternatively, inducible promoters could be used to drive the expression of the inserted gene when necessary. As noted, shear stress inducible promoters may be used. Other

inducible promoters include, but are not limited to, metallothionein and heat shock protein.

Examples of transcriptional control regions that exhibit tissue specificity for connective tissues which have been described and could be used, include but are not limited to: elastin or elastase I gene control region which is active in pancreatic acinar cells (Swit et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515). The deposition of elastin is correlated with specific physiological and developmental events in different tissues, including vascular grafts. For example, in developing arteries, elastin deposition appears to be coordinated with changes in arterial pressure and mechanical activity. The transduction mechanisms that link mechanical activity to elastin expression involve cell-surface receptors. Once elastin-synthesizing cells are attached to elastin through cell-surface receptors, the synthesis of additional elastin and other matrix proteins may be influenced by exposure to stress or mechanical forces in the tissue (for example, the constant movement of the construct in the bioreactor) or other factors that influence cellular shape.

In yet another embodiment of the invention, the three-dimensional culture system could be used in vitro to produce biological products in high yield. For example, a cell which naturally produces large quantities of a particular biological product (e.g., a growth factor, regulatory factor, peptide hormone, antibody, etc.), or a host cell genetically engineered to produce a foreign gene product, could be clonally expanded using the three-dimensional culture system in vitro. If the transformed cell secretes the gene product into the nutrient medium, the product may be readily isolated from the spent or conditioned medium using standard separation techniques (e.g., HPLC, column chromatography, electrophoretic techniques, to name but a few). A bioreactor has been devised which takes advantage of the flow method for feeding the three-dimensional cultures in vitro. Essentially, as fresh media is passed through the three-dimensional culture, the gene product is washed out of the culture along with the cells released from the culture. The

gene product is isolated (e.g., by HPLC column chromatography, electrophoresis, etc.) from the outflow of spent or conditioned media.

In a further embodiment of the invention, three-dimensional cultures maybe used to facilitate gene transduction. For example, and not by way of limitation, three-dimensional cultures of fibroblast stroma comprising a recombinant virus expression vector may be used to transfer the recombinant virus into cells brought into contact with the stromal matrix, thereby simulating viral transmission in vivo. The three-dimensional culture system is a more efficient way of accomplishing gene transduction than are current techniques for DNA transfection.

The use of the genetically engineered three-dimensional cultures of this invention in gene therapy has a number of advantages. Firstly, since the culture comprises eukaryotic cells, the gene product will be properly expressed and processed in culture to form an active product. Secondly, gene therapy techniques are useful only if the number of transfected cells can be substantially enhanced to be of clinical value, relevance, and utility; the three-dimensional cultures of the invention allow for expansion of the number of transfected cells and amplification (via cell division) of transfected cells.

6. EXAMPLE

6.1 ISOLATION AND CULTURING OF VASCULAR SMOOTH MUSCLE CELLS

Canine vascular SMCs were isolated from the right coronary artery by collagenase and elastase digestion as described, e.g., by Redmond et al., 1995, In Vitro Cell Dev. Biol. 31: 601-609. Briefly, the coronary artery was surgically removed from an anesthetized animal and aseptically rinsed using an antibiotic/antimycotic solution (1X antibiotic/antimycotic solution, Gibco, Gaithersburg MD). The adventitia was excised from the artery using a sterile forceps, scissors or scalpel and rinsed with 1X antibiotic/antimycotic solution. The medial tissue of the vessel was placed in a solution of 1X Trypsin EDTA (Gibco)

and 0.2% collagenase IV and incubated on a rotator for 15 min at 37°C. The medial tissue was minced into small pieces, placed in a prewarmed 37°C solution of 0.25 mg/ml elastase, 0.7 mg/ml collagenase I, 0.4 mg/ml soy trypsin inhibitor (Worthington Biochem) and 0.1 mg/ml BSA in PBS (phosphate buffered saline) lacking calcium and magnesium and incubated for 1 hr. at 37°C with rotation. The supernatant was collected and the digested tissue was rinsed with DMEM (Dulbecco's modified eagle medium, Gibco) supplemented with 20% FBS (fetal bovine serum; Hyclone, Logan UT). The medium was removed and combined with the collected supernatant. The cells present in the combined supernatant were collected by centrifugation at 1200 rpm for 10 min. The cell pellet was resuspended in DMEM supplemented with 20% FBS and placed in growth flasks for cell culturing.

Isolated cells were cultured in DMEM with 10% FBS, 2mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco) and 1X antibiotic/antimycotic solution. Cells were cultured at 37°C in a humidified incubator with 5% CO_2 and isolated smooth muscle cells were identified by positive immunostaining for α-smooth muscle actin and calponin and negative staining for von Willebrand factor. Purified SMC cultures were passaged for 6 to 8 passages prior to analysis.

6.2 APPLICATION OF SHEAR FLOW STRESS TO ISOLATED SMCs

A parallel-plate flow chamber such as that described by Frangos et al., 1988, Biotech. Bioeng. 32: 1053-1060 was used to apply steady laminar shear flow stress to monolayer SMC cell cultures. Briefly, in this system, flow was driven through the flow chamber by the hydrostatic pressure head created by the vertical distance between the upper and lower fluid reservoirs. The pressure head was kept constant by continuous pumping of culture media through the flow loop system. The parallel plate system consisted of a machined polycarbonate plate, a rectangular silicone elastic gasket, and a glass slide with the attached cultured cell monolayer. The shear stress, τ, on the cell monolayer was determined from the following equation: $\tau = \frac{6Q\mu}{bh^2}$

where Q is flow rate (cm^3/s), μ is viscosity (dynes X sec/cm^2), b is channel entry width, and h is channel height.

In this system, cultured canine SMCs (passage 6-8) were plated onto fibronectin-coated glass slides (2 $\mu g/cm^2$) and monolayer cultures grown to confluence as described above. The slides were attached to the polycarbonate chamber with the gasket and subjected to a steady laminar shear flow stress of 20 $dynes/cm^2$ for up to 72 hours. The recirculating medium consisted of the medium described above supplemented with 50 $\mu g/ml$ ascorbic acid. Photographs of cell monolayers were taken at designated time intervals using a 35-mm camera attached to a phase contrast microscope (Zeiss). Photographs were scanned for subsequent digital image processing using an automated method for measuring orientation in microscopic images (see, e.g., Chaudhuri et al., 1993, Pattern Recognition Letters 14(2): 147-153). Local orientation of the images was determined by examining local intensity gradients in small regions of each image. The algorithm was implemented using the C programming language on a Silicon Graphics O2 workstation (see Chaudhuri et al., supra). The automated image processing method was used to quantify mean angular distributions of cell orientation $\pm$ 90° with respect to flow direction.

6.3 RESULTS

Exposure of the cells to shear flow stress at 20 $dynes/cm^2$ for 48 hours induced SMC alignment in an orientation perpendicular to the direction of flow compared with the random cell orientation in the no-flow control. More specifically, mean orientations determined by the automated image processing method described above for flow versus no-flow conditions were significantly different, -88.4° vs. +37.6°; $p < 0.001$. See, e.g., Figure 1. Furthermore, increased levels of shear flow stress produced significantly greater cell alignment perpendicular to the direction of flow compared to static controls, 10.7° vs. 32.9°; $p < 0.0001$. See, e.g., Figure 2. The histograms of Figure 2 confirm that as applied shear stress increases, the distribution of cell orientation angle changes from a random pattern to a distribution centered around -90° and +90°,

indicating perpendicular alignment of the cells relative to the direction of flow. Thus, these experiments demonstrate that applying steady laminar shear flow stress to cultured SMCs causes alignment of the cells in an orientation that is perpendicular to the direction of flow.

In addition, the effect of shear stress on SMC microfilament alignment was visualized via fluorescent staining of F-actin filaments as follows: after experimentation as described above, the SMC monolayers were washed in PBS at 37°C and fixed in 3.7% formaldehyde. Cells were then permeabilized with 0.1% Triton-X 100 and stained with Oregon Green 488-phalloidin (Molecular Probes, Eugene OR. Fluorescent staining of the F-actin microfilaments also showed perpendicular orientation of the cells under flow.

A further study was undertaken to determine the effect of duration and magnitude of shear stress on SMC cellular alignment. SMC cells were grown in monolayer as described above and shear flow stress was applied to the cells using the apparatus described above at different magnitudes and durations of stress. Images of the cell monolayers undergoing shear stress were taken with a CCD camera and recorded with a time lapse VCR.

The results of this study are indicated in Figure 3. In fact, the speed of perpendicular alignment of the SMCs under shear flow stress was dependent on the magnitude and duration of the stress. As indicated, for example, in Figure 3, cells exposed to 20 dynes/cm^2 aligned perpendicularly more quickly, i.e., within 24 hours, compared to cells exposed to 15 dynes/cm^2, which aligned within 48 hours. In addition, as depicted in Figure 4, SMC monolayers exposed to 20 dynes/cm^2 began aligning perpendicular to the flow within hours of the onset of the shear flow stress. Image processing of time lapse video images revealed that the angular variance of cell orientation angle decreased dramatically by 8 hours of shear flow stress exposure, indicating an increase in cellular alignment.

Additional studies determined that the perpendicular alignment of the SMCs under shear flow stress was inhibited by the addition of the calcium chelator, quin2-AM, 31° vs. 14°; $p < 0.02$. More specifically, SMC monolayers were treated

for 1 hr. with 10 μM quin2-AM in DMSO. The cells were then exposed to shear flow stress using medium containing 1 μM quin2-AM and the automated image processing method described above was used to quantify the direction and degree of cell alignment, where angular deviations less than 20° indicate significant alignment. As noted, treatment with quin2-AM inhibited shear-induced SMC alignment when compared with non-treated cells (see Figure 5A-5B).

Further, to determine the role of microtubule organization in SMC alignment, SMC monolayers were treated with 3 μM nocodazole (in DMSO), a known inhibitor of microtubule networks, for 24 hours prior to the application of shear flow stress. During the application of shear flow stress 3 μM nocodazole was added to the circulating medium. Nocodazole treatment was found to obstruct SMC alignment under fluid flow, 33° vs. 18°; $p < 0.005$. These results, depicted in Figure 6A-6B, demonstrate that intracellular calcium and the microtubule network may be important in the regulation of SMC alignment by fluid shear stress.

6.4 SHEAR STRESS ALIGNMENT OF CELLS IN THREE-DIMENSIONAL CULTURE

Shear flow stress has also been utilized to align smooth muscle cells perpendicular to the direction of flow in three-dimensional cultures.

According to one embodiment of the invention, sterile porous tubular polymer scaffolds can be briefly dipped in filtered 70% ethanol to wet the polymer surface and then soaked in culture medium for 24 hr. The culture medium consists of DMEM containing 10% FBS, 2mM L-glutamine, 0.1 mM non-essential amino acids, and 1X antibiotic/antimycotic solution. Adhesion of serum proteins to the scaffold enhances subsequent cell attachment. Each scaffold can then be placed in a 15 ml conical tube containing a suspension of 10^6 vascular SMCs in 13 ml of medium. The conical tubes can be rotated for 24 hr. at 37°C, resulting in uniform cell attachment throughout the scaffolds.

According to another embodiment, porous tubular polyurethane scaffolds were fabricated using a solvent-cast/particulate-leaching method as described by Thomson et

al., Polymer Scaffold Processing. In *Principles of Tissue Engineering*, RP Lanza, R. Langer and WL Chick (eds.), pp. 263-272 (RG Landes Co, Austin, TX). This procedure is also described in United States Patent No. 5,514,378. Briefly, polyurethane (Tecothane; Thermedics, Inc.; Woburn, MA) was dissolved in tetrahydrofuran (Sigma # T-5267; St. Louis, MO) solvent and then mixed with sodium chloride (NaCl) (Sigma # S9888) particles between 150-300 μm at a 90% void fraction ratio (salt:polymer weight) to create pore sizes of the same size. The polymer/salt mixture was repeatedly dip cast onto 4-mm diameter rods to create tubular structures. The structures were then allowed to air dry overnight and the NaCl was leached in several changes of deionized water. After drying, the scaffolds were cut to 6-cm lengths, luers were attached to each end, and scaffolds were placed into separate conical tubes for electron beam sterilization (2.0 Mrad). Sterilized scaffolds were rewetted in 100% and 70% ethanol and rinsed in two changes of phosphate buffered saline (Gibco # 14190-136). To prepare the surfaces for cell attachment, scaffolds were soaked overnight in 10 μg/ml of sterile-filtered fibronectin (Sigma # F-1141) in an incubator (37°C, 5% CO_2, 90% relative humidity).

Next, the fibronectin-coated scaffolds were aspirated semi-dry and placed in a high density suspension of passaged canine coronary artery smooth muscle cells (20 X 10^6 cells/ml) (isolated as described in Section 6.1, supra) and DMEM (Gibco #11960-051) for approximately 30 seconds (approximately 0.5 ml or 10 X 10^6 cells fill the pores of the graft). Seeded grafts were placed into empty 15-ml conical tubes, capped tightly, and rotated circumferentially (approximately 1 rpm) for 4 hours in an incubator. The grafts were placed in DMEM containing 20% fetal bovine serum (Hyclone, # SH30070.03), 3 ng/ml cupric sulfate (Sigma # C-8027), 1% antibiotic/antimycotic (Gibco # 15240-062), 1% non-essential amino acids (Gibco # 11140-050), 1% L-glutamine (Gibco #25030-081), and 1% sodium pyruvate (Gibco # 11360-070) and capped loosely for static growth overnight in an incubator.

The seeded grafts were next cultured in fluid flow bioreactors, e.g., as described in United States Patent No.

5,792,603, under steady flow. The bioreactor permits adjustable fluid flow through the lumens of the three-dimensional structures, thereby providing control of shear stress at the luminal wall.

More specifically, the grafts were placed in 50 ml fluid flow bioreactors with approximately 50 ml of the above medium formulation supplemented with 50 μg/ml L-ascorbic acid (Sigma # A-4034). The grafts were cultured with fluid flowing through the lumen at approximately 0.3 dyne/cm² fluid shear stress. The ascorbate-supplemented medium was changed weekly (every 5-7 days) for 3-5 weeks. Alternatively, partial media changes (e.g., 50%) can be performed weekly to provide fresh nutrients while retaining autocrine factors produced by the cells (e.g., growth factors).

The grafts cultured as described above, also referred to herein as tissue-engineered vascular grafts (TEVGs), demonstrated good attachment and growth of the smooth muscle cells on the grafts. Moreover, scanning electron micrographs showed smooth muscle tissue on the grafts with the smooth muscle cells aligned perpendicular to the direction of flow (see Figure 11). Thus, the present experiments demonstrated that the seeding of smooth muscle cells on three-dimensional tubular vascular grafts and subsequent culturing of the cells/grafts under shear flow stress results in the alignment of the cells in an orientation that is perpendicular to the direction of flow, an alignment that is analogous to that displayed by SMCs in vivo, e.g., in native blood vessels.

In a further embodiment of the invention, canine microvascular endothelial cells (MVEC) were seeded onto the TEVGs described above and subjected to shear flow stress. More specifically, canine MVEC were isolated from canine falciform ligament fat as described, e.g., by Williams SK, Jarrell BE, Kleinert LB., 1994 (Nov-Dec), J Invest Surg 7(6):503-17 and 40,000 to 60,000 cells/cm² were seeded into the lumen of the TEVGs by passive seeding. Alternatively, the cells can be seeded by forced deposition. In addition, the endothelial cells can be optionally seeded at a density of less than 50% confluency.

After seeding, the TEVGs were placed into bioreactors as described above and rotated for 4 to 8 hours at 1 rpm. The

bioreactors were attached to peristaltic pumps and medium was pumped at a fluid shear stress of approximately 0.1 dynes/cm² at a steady laminer flow. After 3 days, the flow was increased to approximately 6 dynes/cm² (steady laminar flow) and the TEVGs were cultured for an additional 3 days.

Alternatively, human MVEC can be used in this embodiment; such MVECs can be obtained commercially at passage 3 from Cascade Biologics, Inc., Portland, OR, and cultured in Medium 131 (Cascade) containing Microvascular Growth Supplement (MVGS, Cascade). Cells at passage 5 can be harvested with Trypsin/EDTA (Gibco, Rockville MD), resuspended to 7.5×10^5 cells/ml in supplemented Medium 131, and 0.7 ml seeded into the lumen of each TEVG.

The TEVG grafts of this embodiment were characterized for MVEC presence and orientation using fluorescently-labeled proteins that interact with endothelial cells such as acetylated low density lipoprotein (Molecular Probes, Eugene, OR) or antibodies against CD31 (Pharmigen, San Diego), an antigen expressed on endothelial cells, followed by scanning electron microscopy.

As indicated in Figure 12, the vascular endothelial cells that were seeded onto the SMC-containing TEVGs attached well under shear flow stress to the luminal wall of the grafts, forming a smooth endothelial lining with the cells aligned parellel to the direction of flow. Good attachment was obtained at a shear flow stress of about 6 dynes/cm². Control grafts cultured under very low shear flow stress, e.g., about 0.1 dynes/cm² did not demonstrate endothelial cell alignment (data not shown).

The alignment of the endothelial cells parallel to the direction of flow in these experiments is analogous to the orientation of such cells in vivo, e.g., in native blood vessels (see, e.g., Ballerman et al., WO 94/25584). Thus, the three-dimensional culture of the invention wherein SMCs are grown under shear flow stress and align perpendicular to the direction of flow and then endothelial cells are seeded onto the cultures and align parallel to the direction of flow along the luminal wall of the cultures results in a structure that is very similar in morphology to that seen in vascular structures in vivo.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

We claim:

1. A method for culturing smooth muscle cells in vitro comprising growing stromal cells comprising smooth muscle cells on a substrate under shear flow stress such that the smooth muscle cells align perpendicular to the direction of the shear flow.

2. A method for culturing smooth muscle cells in vitro comprising:

a) culturing stromal cells comprising smooth muscle cells on a substrate; and

b) subsequently subjecting the cultured cells to shear flow stress such that the smooth muscle cells align perpendicular to the direction of the shear flow.

3. The method of claim 1 or 2, wherein the substrate is a surface that supports the growth of the smooth muscle cells in a monolayer.

4. The method of claim 1 or 2, wherein the substrate is a scaffold that supports the growth of the smooth muscle cells in a three-dimensional culture.

5. The method of claim 4, wherein the stromal cells comprising smooth muscle cells are cultured on a three-dimensional scaffold composed of a biocompatible non-living material having interstitial spaces that are bridged by the stromal cells.

6. The method of claim 1 or 2, wherein the stromal cells additionally comprise fibroblasts, endothelial cells or other loose connective tissue cells.

7. The method of claim 5, wherein the stromal cells additionally comprise fibroblasts, endothelial cells or other loose connective tissue cells.

8. The method of claim 1 or 2, wherein the shear flow stress is in the range of about 0.1 to about 100 dynes/cm^2.

9. The method of claim 5, wherein the shear flow stress is in the range of about 0.1 to about 100 dynes/cm^2.

10. The method of claim 1 or 2, wherein the shear flow stress is in the range of about 0.3 to about 50 dynes/cm^2.

11. The method of claim 5, wherein the shear flow stress is in the range of about 0.3 to about 50 dynes/cm^2.

12. The method of claim 1 or 2, wherein the shear flow stress is in the range of about 0.3 to about 25 dynes/cm^2.

13. The method of claim 5, wherein the shear flow stress is in the range of about 0.3 to about 25 dynes/cm^2.

14. The method of claim 5, wherein the scaffold is biodegradable.

15. The method of claim 5, wherein the scaffold is non-biodegradable.

16. The method of claim 15, wherein the scaffold is comprised of polyurethane.

17. The method of claim 16, wherein the polyurethane scaffold has a void fraction ranging from about 80% to about 95%.

18. The method of claim 5, wherein the scaffold has a pore size ranging from about 150-300 μm.

19. The method of claim 1 or 2, wherein the stromal cells are mammalian.

20. The method of claim 5, wherein the stromal cells are mammalian.

21. The method of claim 1 or 2, wherein the stromal cells contain at least one exogenous gene under the control of an expression element.

22. The method of claim 21, wherein the expression element is inducible by shear flow stress.

23. The method of claim 5, wherein the stromal cells contain at least one exogenous gene under the control of an expression element.

24. The method of claim 23, wherein the expression element is inducible by shear flow stress.

25. The method of claim 1 or 2, further comprising subsequently inoculating endothelial cells onto the aligned smooth muscle cells and culturing the endothelial cells under shear flow stress such that the endothelial cells align parallel to the direction of the shear flow.

26. The method of claim 25, wherein the endothelial cells are inoculated at a density of less than 50% confluency.

27. The method of claim 25, wherein the endothelial cells contain at least one exogenous gene under the control of an expression element.

28. The method of claim 27, wherein the expression element is inducible by shear flow stress.

29. The method of claim 25, wherein the stromal cells contain at least one exogenous gene under the control of an expression element.

30. The method of claim 29, wherein the expression element is inducible by shear flow stress.

31. The method of claim 5, further comprising subsequently inoculating endothelial cells onto the aligned

smooth muscle cells and culturing the endothelial cells under shear flow stress such that the endothelial cells align parallel to the direction of the shear flow.

32. The method of claim 31, wherein the endothelial cells are inoculated at a density of less than 50% confluency.

33. The method of claim 31, wherein the endothelial cells contain at least one exogenous gene under the control of an expression element.

34. The method of claim 33, wherein the expression element is inducible by shear flow stress.

35. The method of claim 31, wherein the stromal cells contain at least one exogenous gene under the control of an expression element.

36. The method of claim 35, wherein the expression element is inducible by shear flow stress.

37. A method for producing an improved tubular structure for implantation in vivo comprising culturing in vitro stromal cells comprising smooth muscle cells under shear flow stress on a tubular three-dimensional scaffold composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, such that the smooth muscle cells align on the scaffold in an orientation perpendicular to the direction of the shear flow.

38. The method of claim 37, further comprising subsequently inoculating endothelial cells onto the smooth muscle cells that are aligned on the tubular structure and culturing the tubular structure under shear flow stress such that the endothelial cells align parallel to the direction of the shear flow.

39. A method for producing an improved tubular structure for implantation in vivo comprising culturing in

vitro stromal cell comprising smooth muscle cells under shear flow stress on a flat scaffold composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, such that the smooth muscle cells align on the scaffold in an orientation perpendicular to the direction of the shear flow, and rolling the scaffold into a tubular structure for implantation in vivo.

40. The method of claim 39, further comprising subsequently inoculating endothelial cells onto the smooth muscle cells that are aligned on the flat scaffold structure and culturing the scaffold structure under shear flow stress such that the endothelial cells align parallel to the direction of the shear flow, prior to rolling the scaffold into a tubular structure.

41. A method for producing an improved tubular structure for implantation in vivo comprising:

(a) culturing in vitro stromal cells comprising smooth muscle cells under shear flow stress on at least a first and second flat rectangular scaffold composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, such that the smooth muscle cells align on the scaffolds in an orientation perpendicular to the direction of the shear flow,

(b) laying the first scaffold on top of at least the second scaffold; and

(c) rolling the scaffolds into a tubular structure for implantation in vivo.

42. The method of claim 41, further comprising inoculating endothelial cells onto the smooth muscle cells that are aligned on the first scaffold and culturing the first scaffold under shear flow stress such that the endothelial cells align parallel to the direction of the shear flow, prior to rolling the scaffolds into a tubular structure.

43. A method for producing an improved tubular structure for implantation in vivo comprising:

(a) culturing in vitro stromal cells comprising smooth muscle cells under shear flow stress on a tubular three-dimensional scaffold composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, such that the smooth muscle cells align on the scaffold in an orientation perpendicular to the direction of the shear flow,

(b) culturing in vitro stromal cells comprising smooth muscle cells under shear flow stress on at least one flat rectangular scaffold of a width slightly larger than the outer circumference of the tubular scaffold and composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, such that the smooth muscle cells align on the flat scaffold in an orientation perpendicular to the direction of the shear flow, and

(c) wrapping the flat rectangular scaffold around the outside of the tubular scaffold to form a tubular structure for implantation in vivo.

44. The method of claim 43, further comprising inoculating endothelial cells onto the smooth muscle cells that are aligned on the tubular structure and culturing the tubular structure under shear flow stress such that the endothelial cells align parallel to the direction of the shear flow, prior to wrapping the flat scaffold around the tubular scaffold.

45. A method for producing an improved tubular structure for implantation in vivo comprising culturing in vitro stromal cells comprising smooth muscle cells under shear flow stress on at least two tubular scaffolds having differing diameters and composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, such that the smooth muscle cells align on each scaffold in an orientation perpendicular to the direction of the shear flow, and inserting the scaffold having the smaller diameter inside the scaffold having the

wider diameter to form a tubular structure for implantation in vivo.

46. The method of claim 45, further comprising inoculating endothelial cells onto the smooth muscle cells that are aligned on the tubular structure having the smaller diameter and culturing the smaller-diameter tubular structure under shear flow stress such that the endothelial cells align parallel to the direction of the shear flow, prior to inserting the smaller-diameter scaffold into the wider-diameter scaffold.

47. A method for producing a tissue construct for the repair or replacement of structures containing smooth muscle cells in vivo comprising culturing in vitro stromal cells comprising smooth muscle cells on a substrate that supports the growth of the cells in monolayer under shear flow stress such that the smooth muscle cells align perpendicular to the direction of the shear flow.

48. A three-dimensional tubular tissue construct prepared in vitro comprising stromal cells comprising smooth muscle cells on a tubular three-dimensional scaffold composed of a biocompatible non-living material having interstitial spaces bridged by the stromal cells, wherein the smooth muscle cells are aligned on the scaffold circumferentially relative to the tubular tissue construct.

49. The tissue construct of claim 48, wherein the stromal cells additionally comprise fibroblasts, endothelial cells or other loose connective tissue cells.

50. The tissue construct of claim 48, wherein the stromal cells contain at least one exogenous gene under the control of an expression element.

FIG.1A

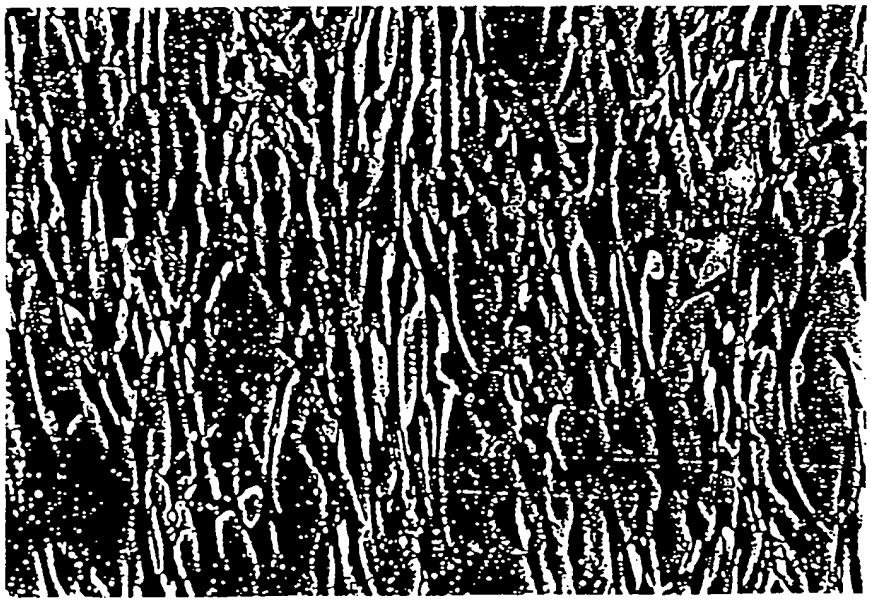
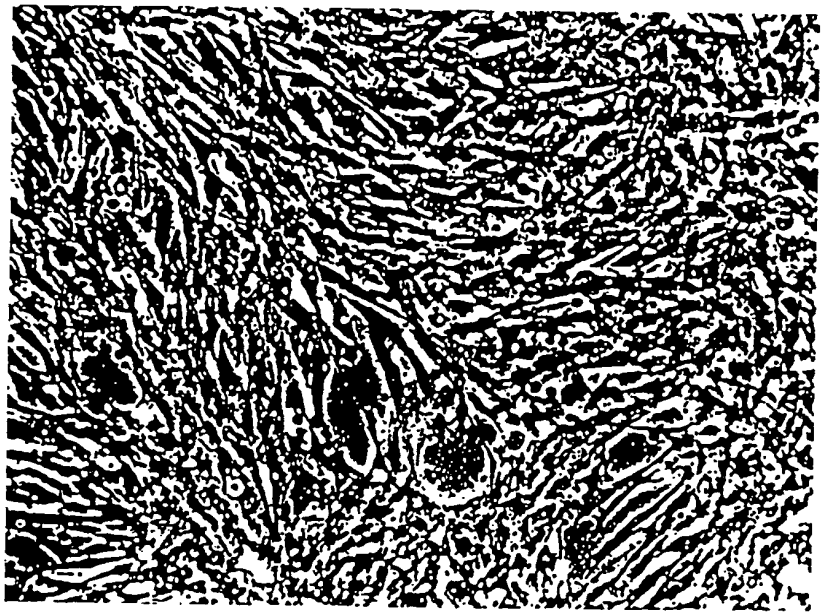


FIG.1B



0 dyn/cm^2

FIG.2A (CONT.)

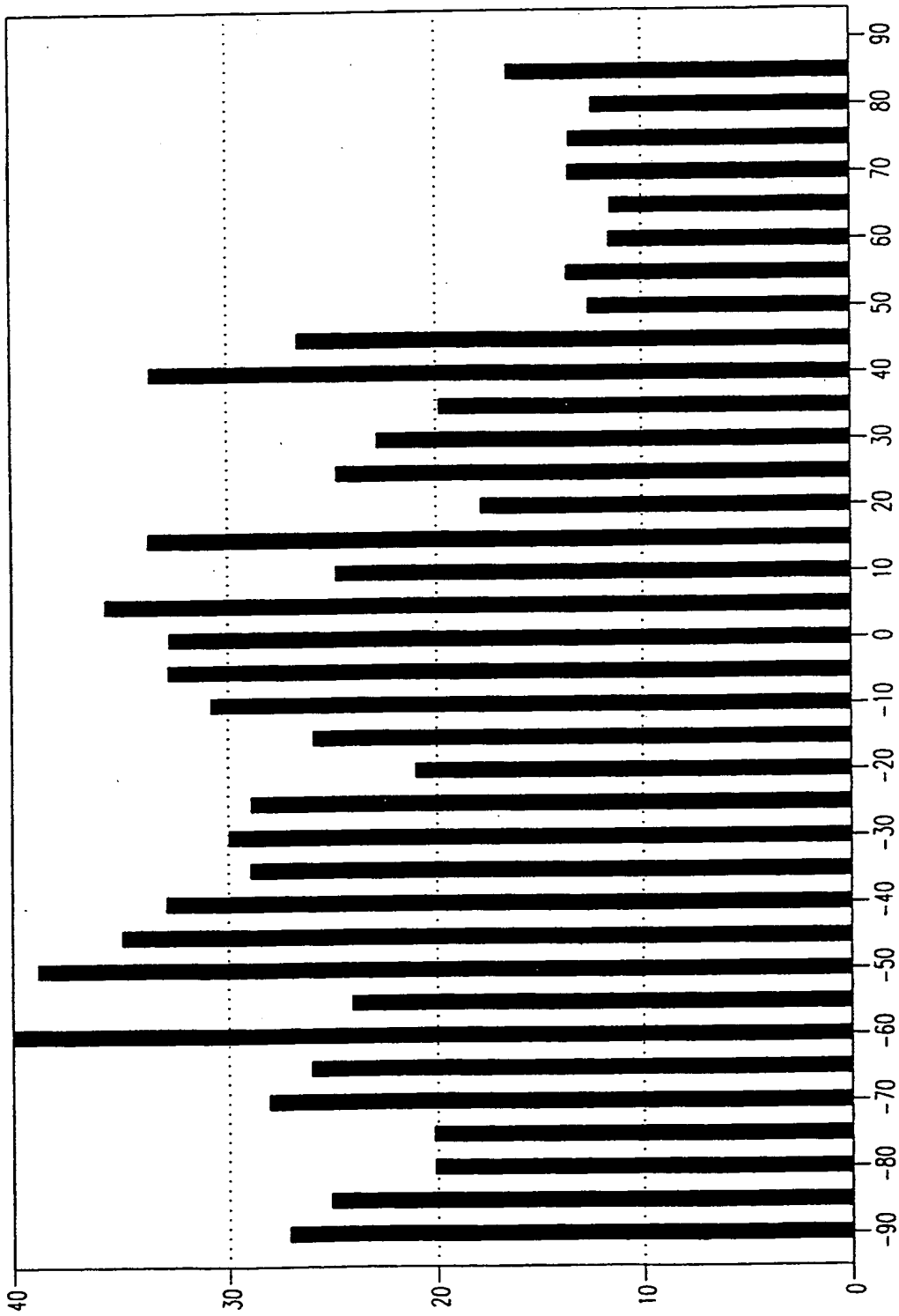

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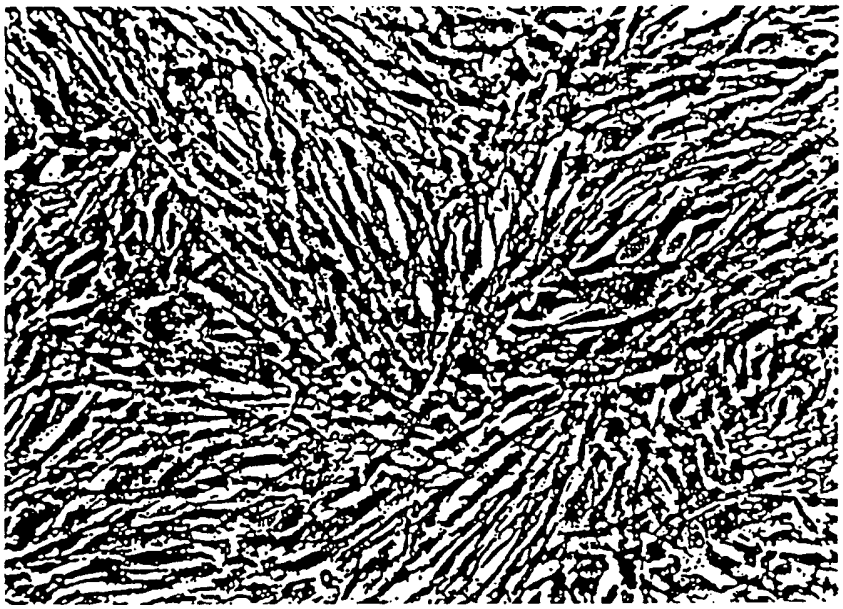
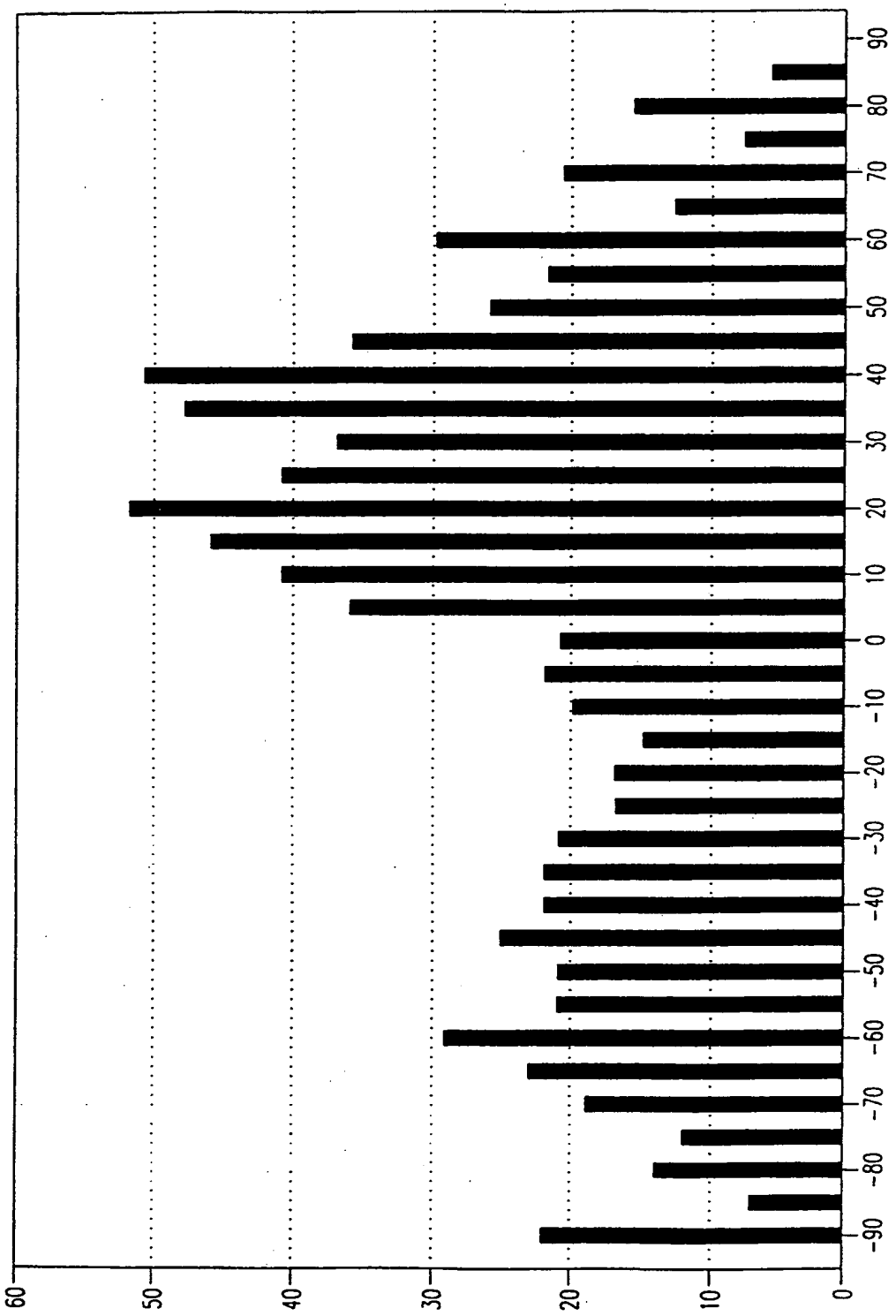
FIG. 2B



1 dyn/cm^2

FIG.2C (CONT.)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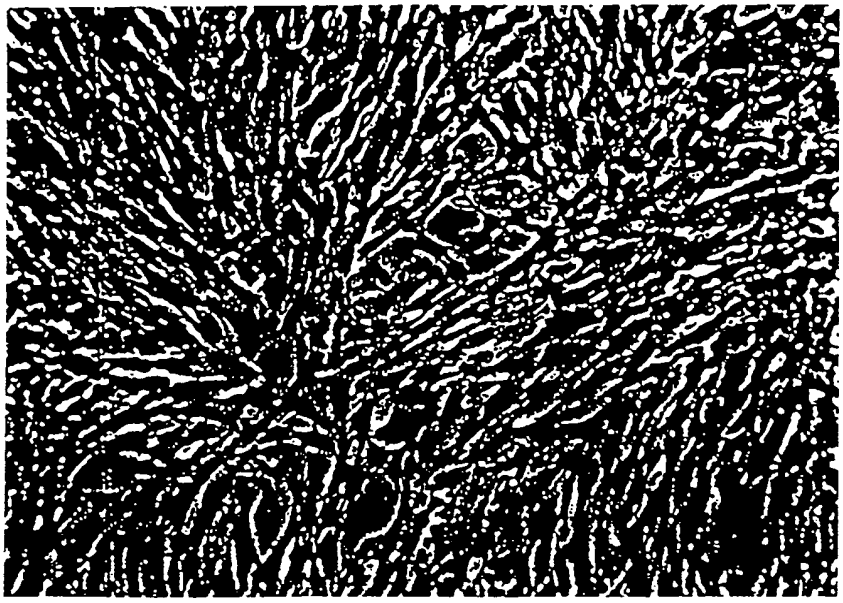
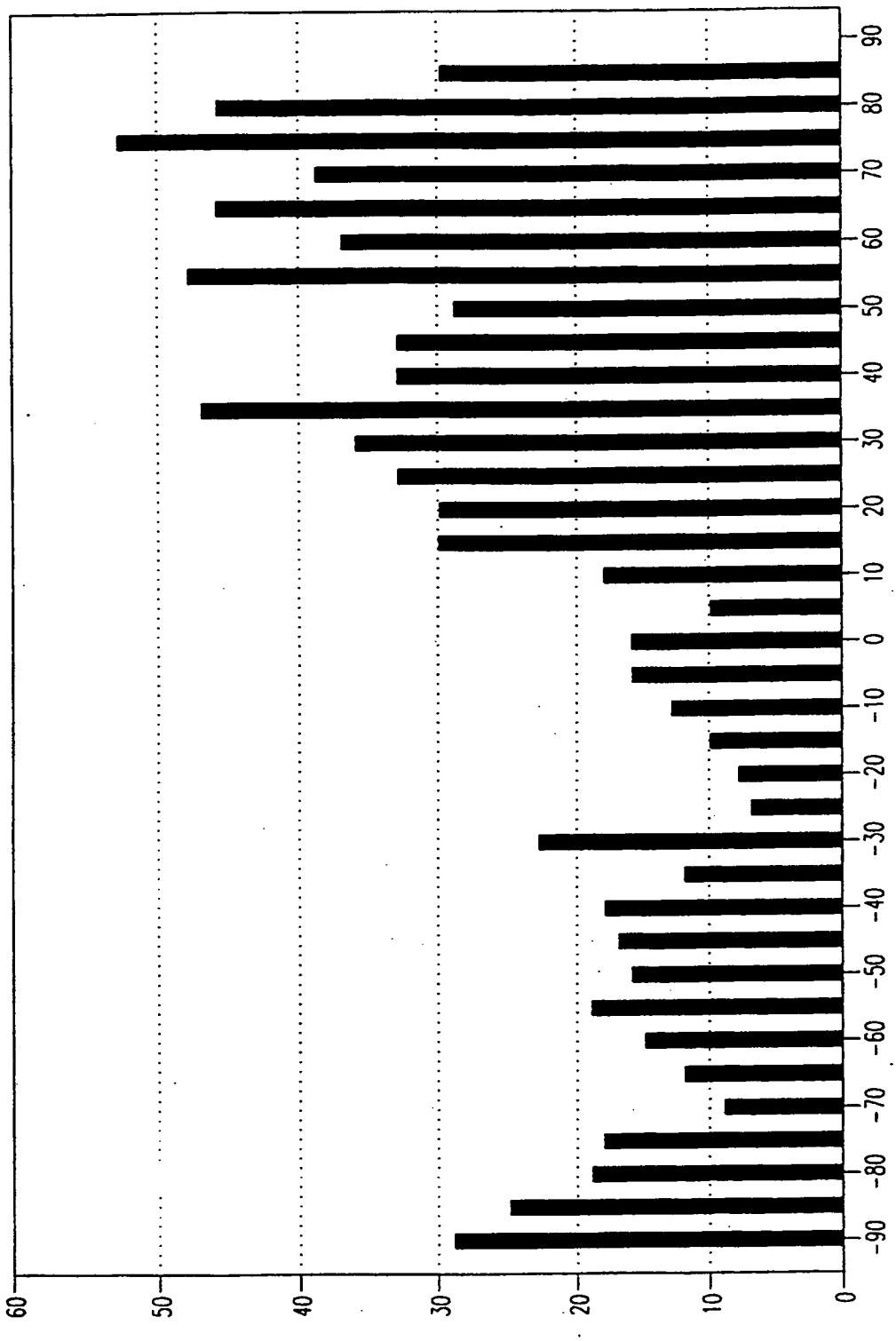
20
10
0
1 dyn/cm2

FIG.2D



10 dyn/cm^2

FIG.2E (CONT.)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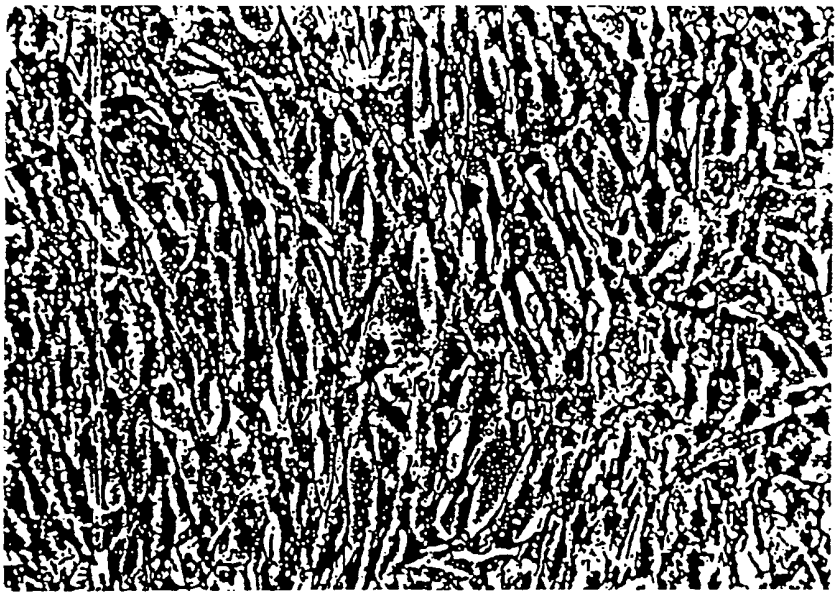
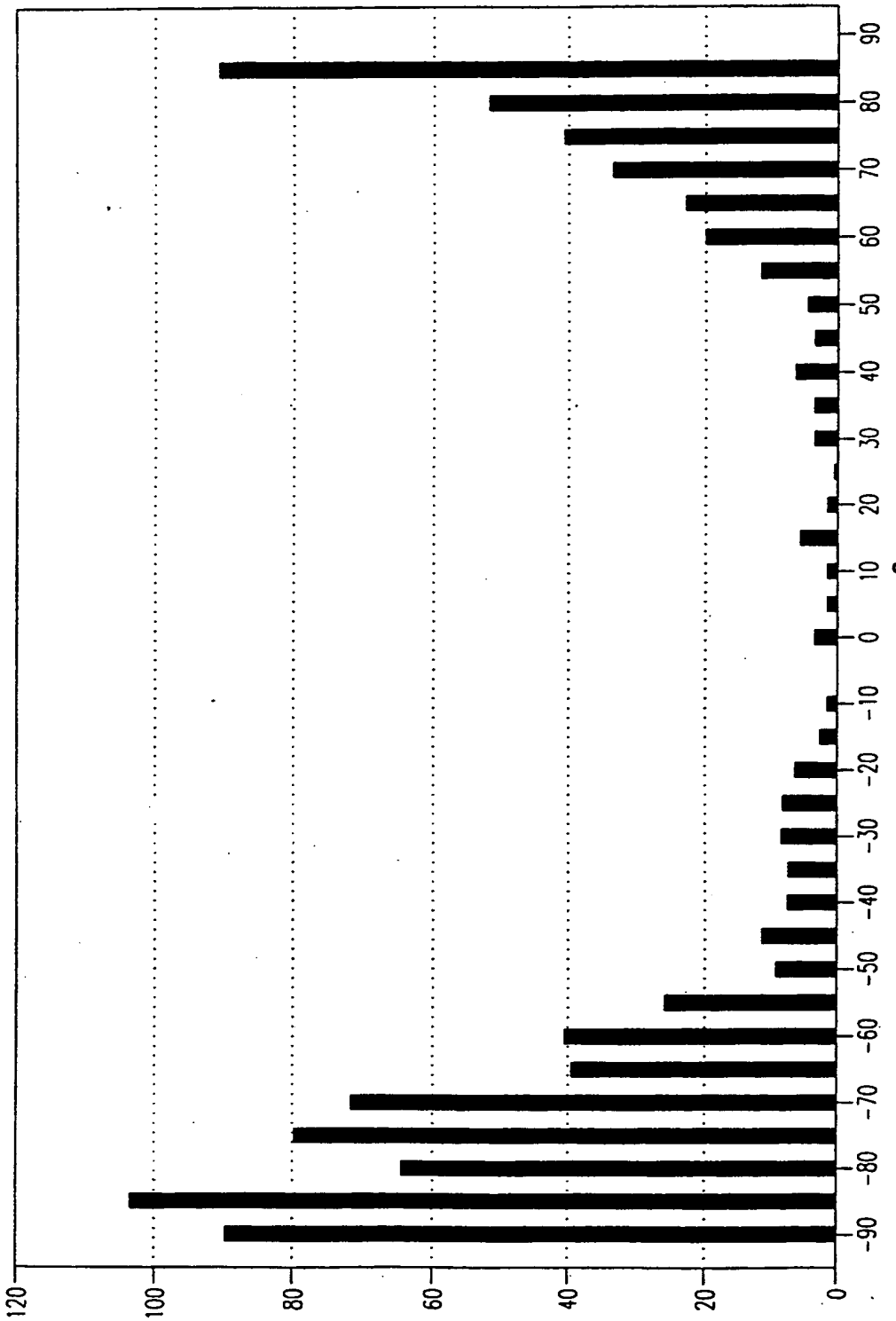
30
20
10
0
10 dyn/cm2

FIG.2F



15 dyn/cm^2

FIG.2G (CONT.)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-90
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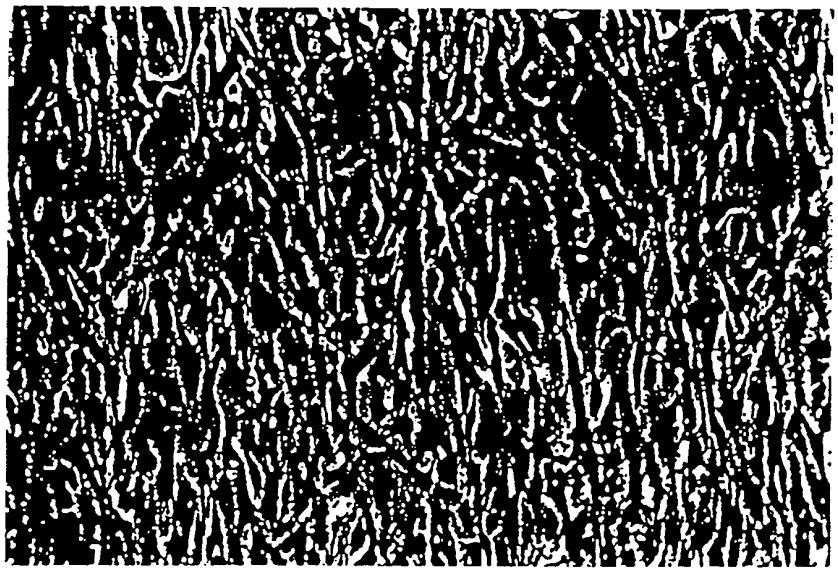
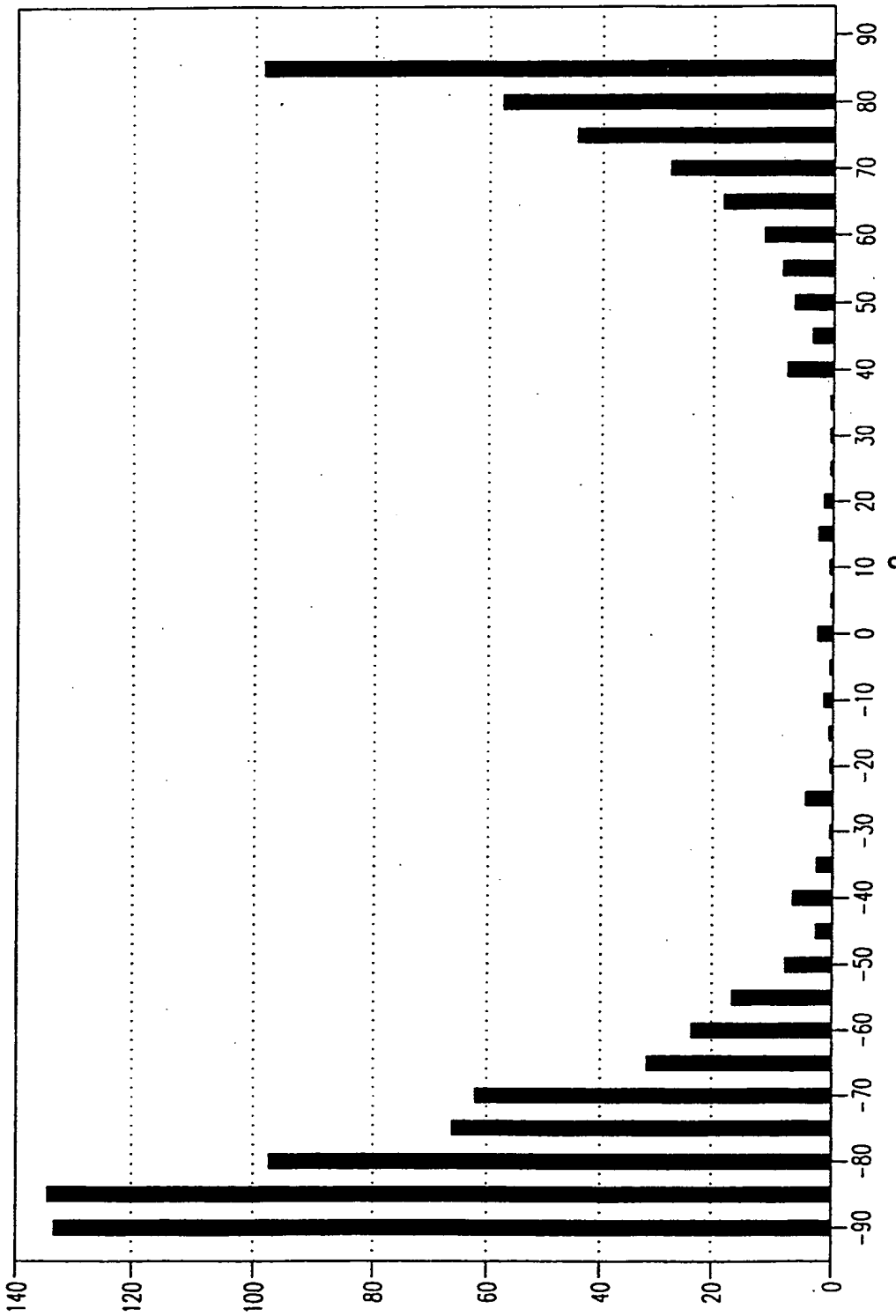
100
80
60
40
20
0
15 dyn/cm2

FIG.2H



20 dyn/cm ^{2}

FIG.2I (CONT.)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-20
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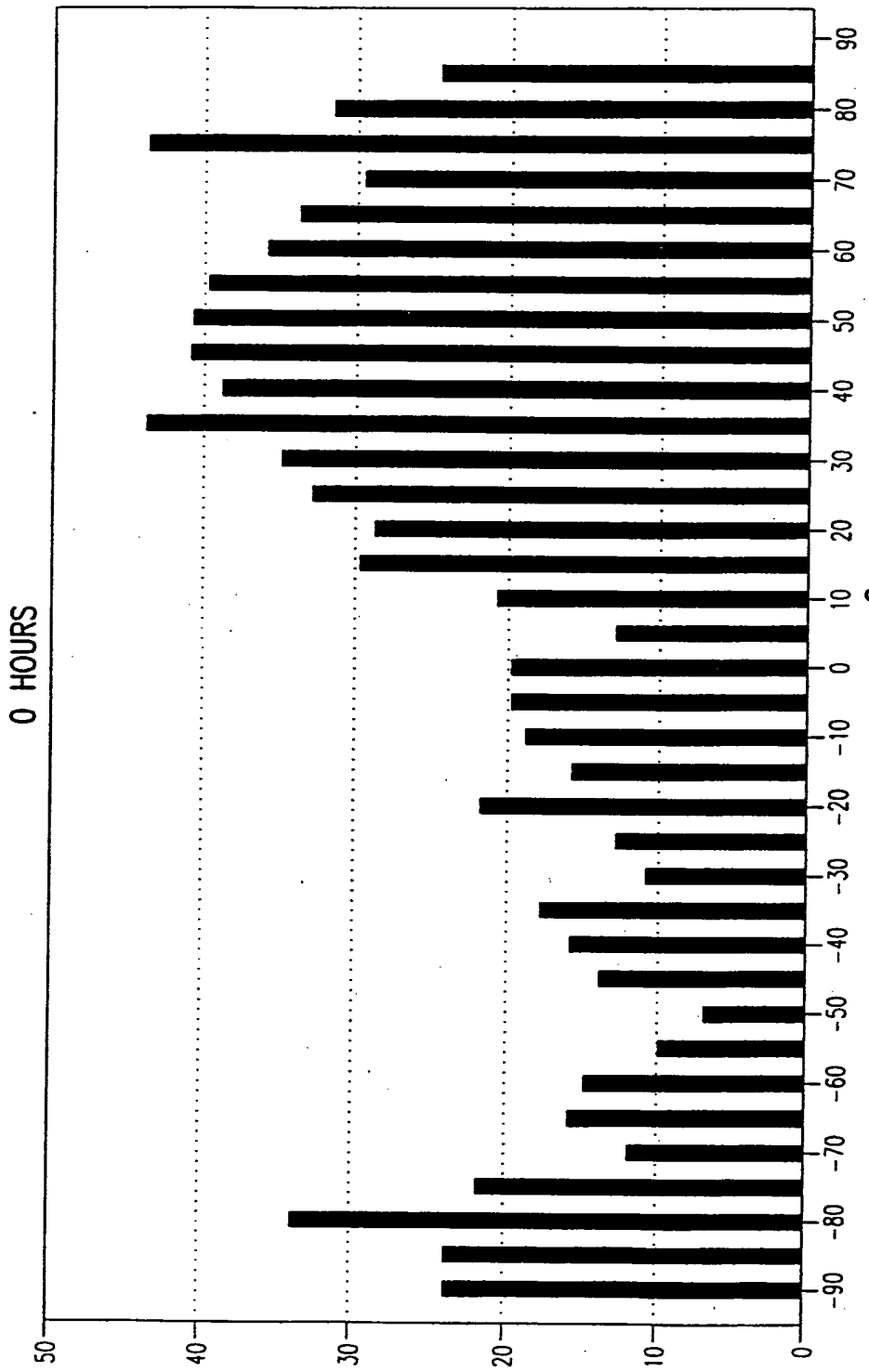
-80
-90
140
120
100
80
60
40
20
0
20 dyn/cm2

FIG.2J


0 HOURS
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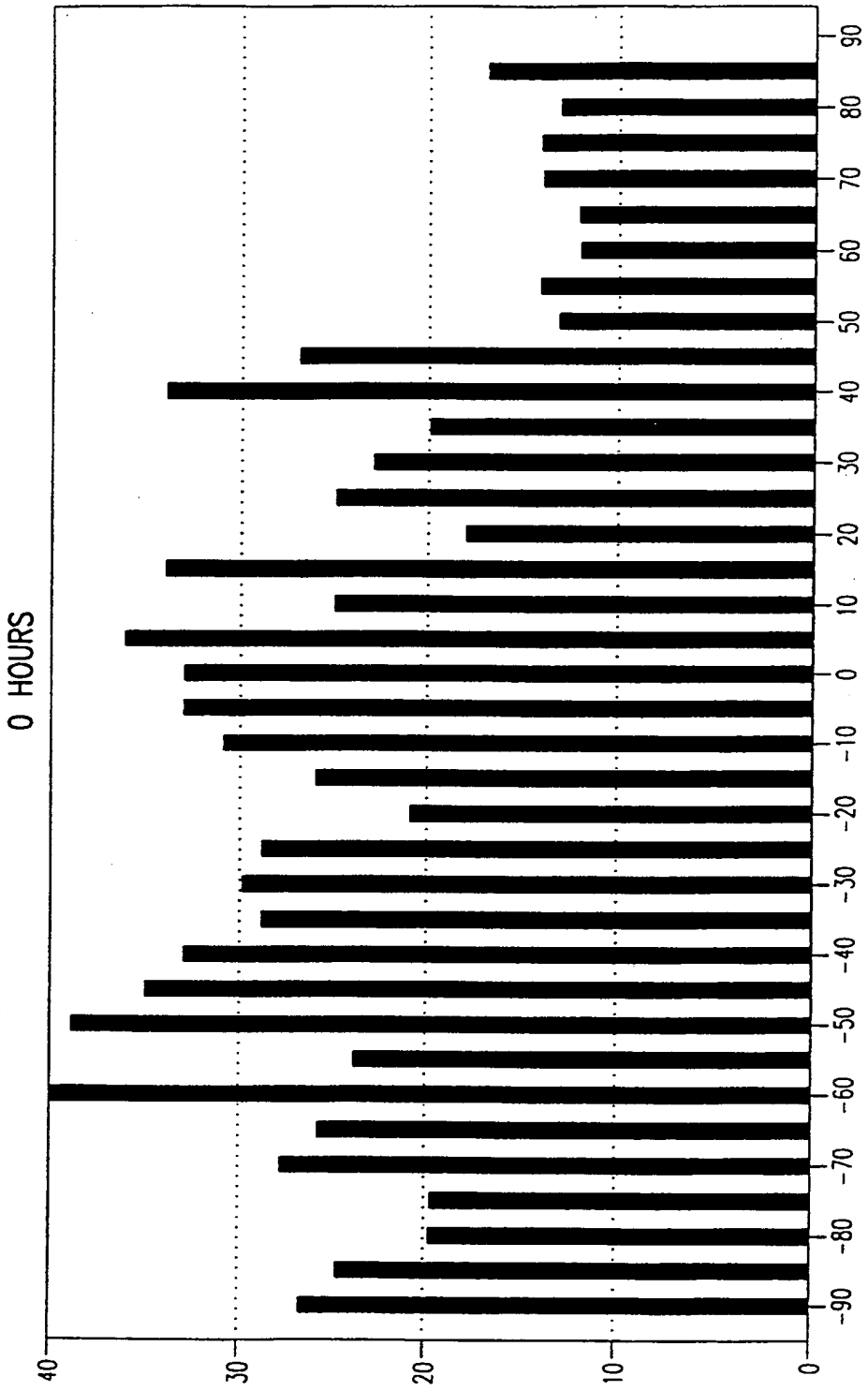
40
30
20
10
0
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
10 dyn/cm2

FIG.3A


0 HOURS
15 dyn/cm2
90
80
70
60
50
40
30
20
10
0
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-80
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40
30
20
10
0

FIG.3B

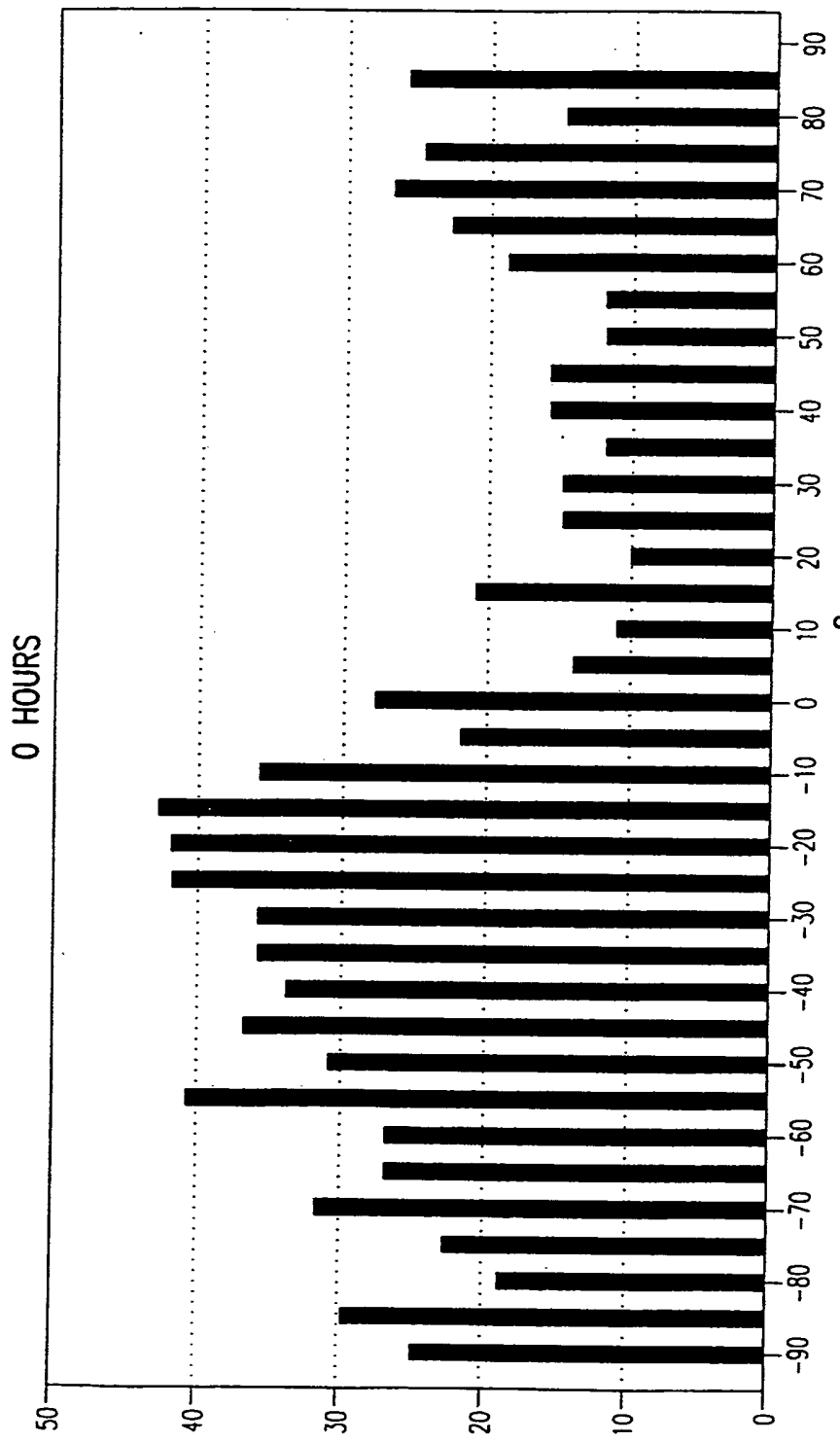

0 HOURS
50
40
30
20
10
0
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
20 dyn/cm2

FIG.3C

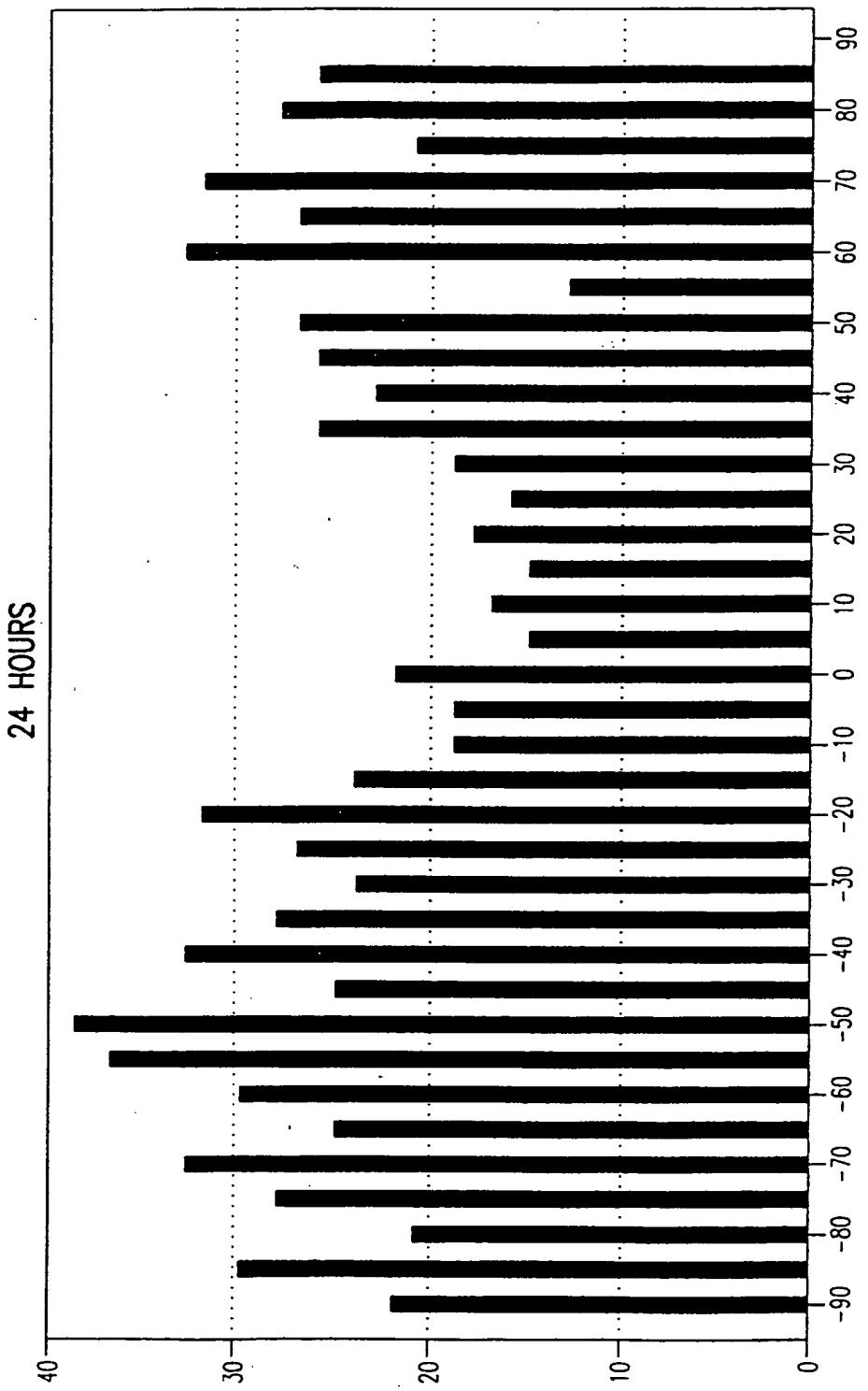

24 HOURS
40
30
20
10
0
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
10 dyn/cm2

FIG.3D

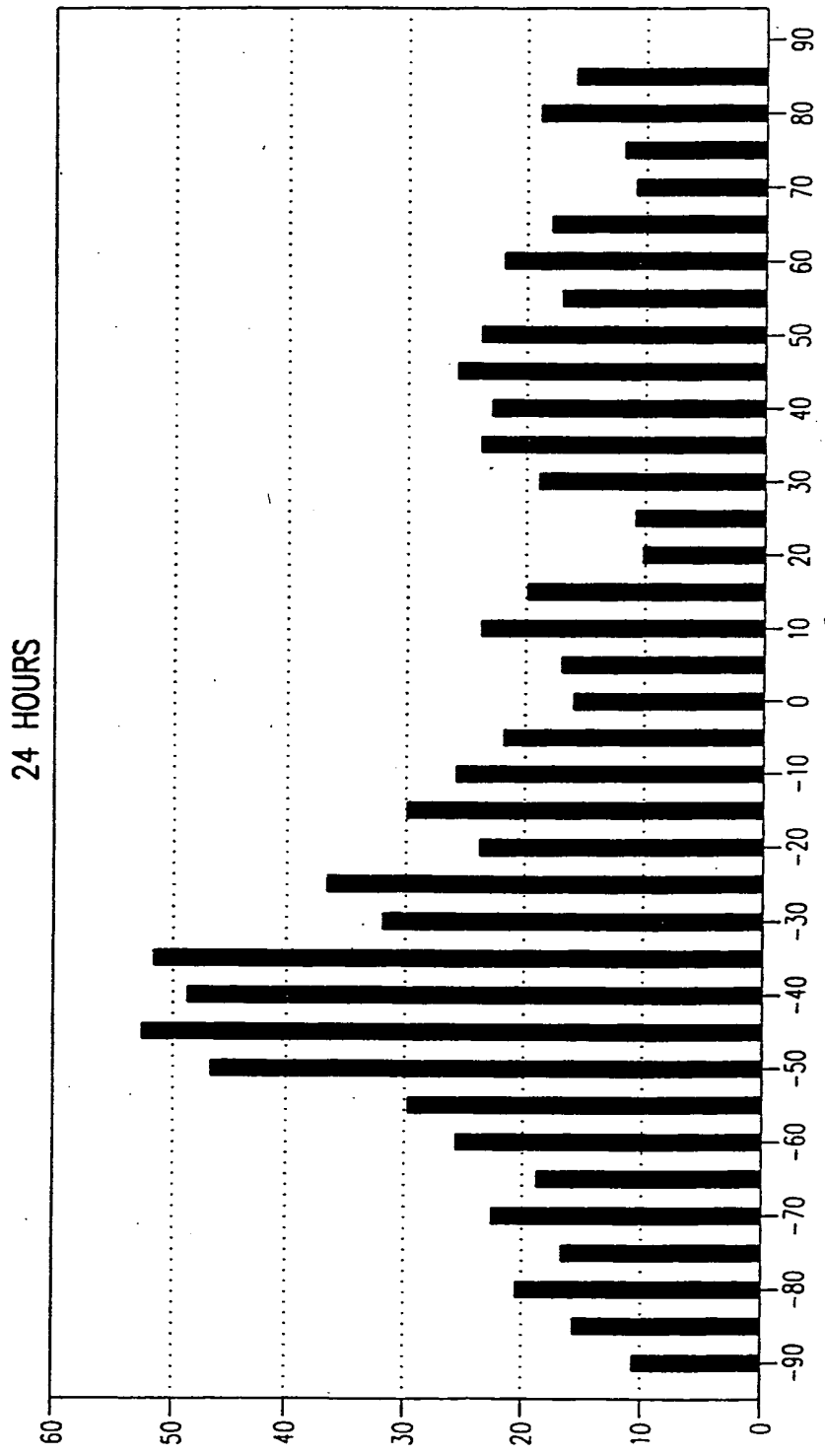

24 HOURS
15 dyn/cm2
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
60
50
40
30
20
10
0

FIG.3E

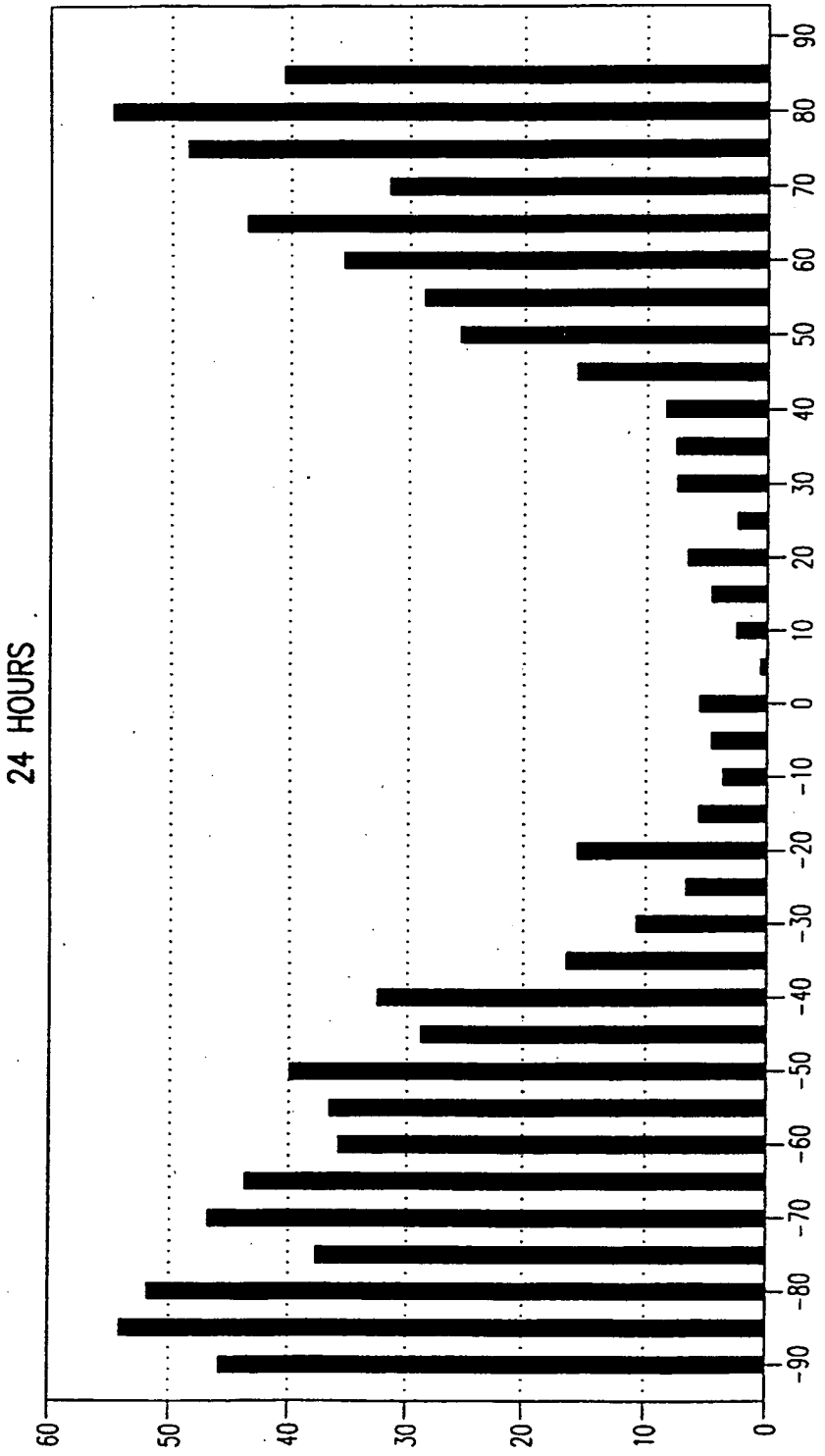

24 HOURS
20 dyn/cm2
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
60
50
40
30
20
10
0

FIG.3F

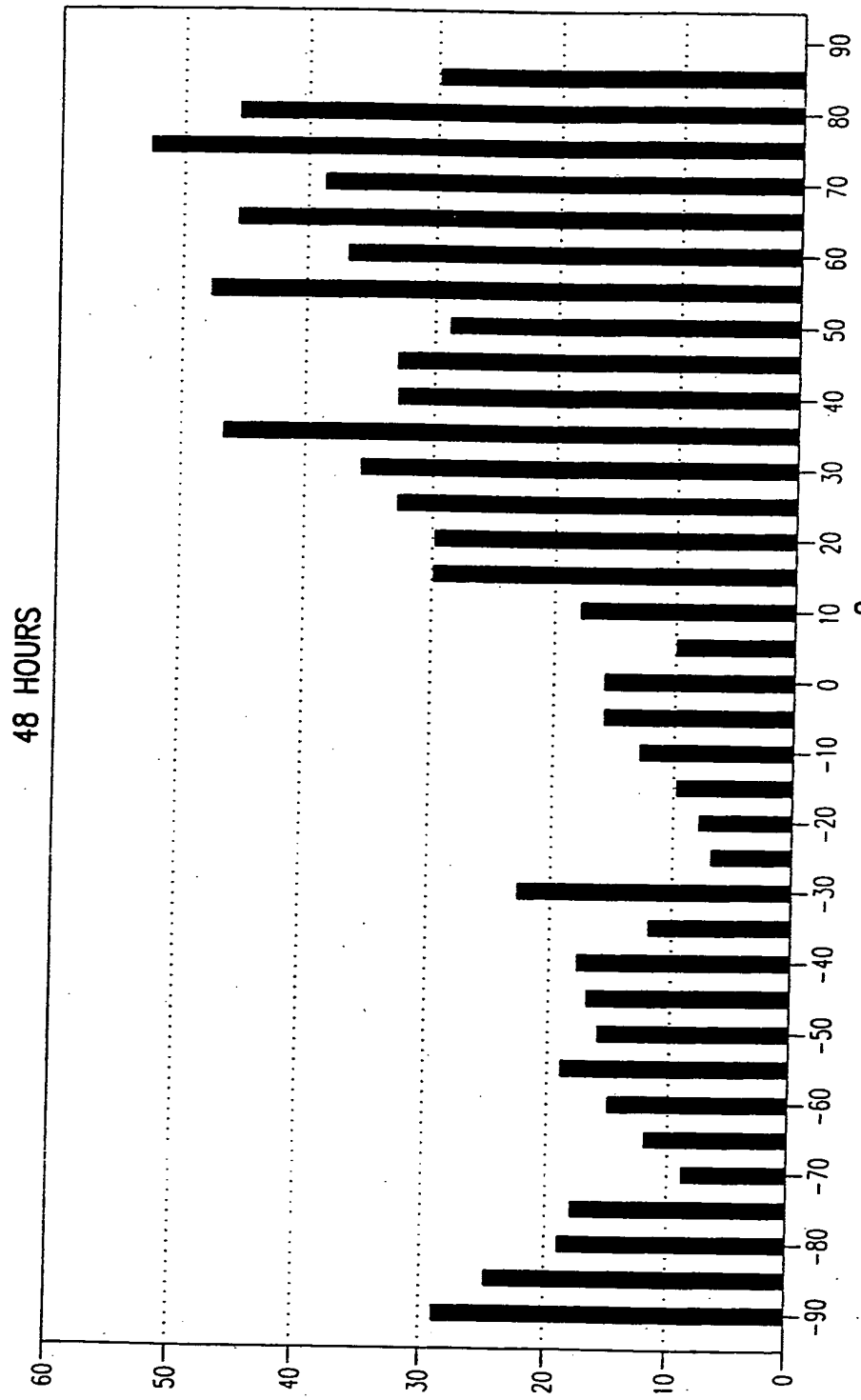

48 HOURS
10 dyn/cm2
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
60
50
40
30
20
10
0

FIG.3G

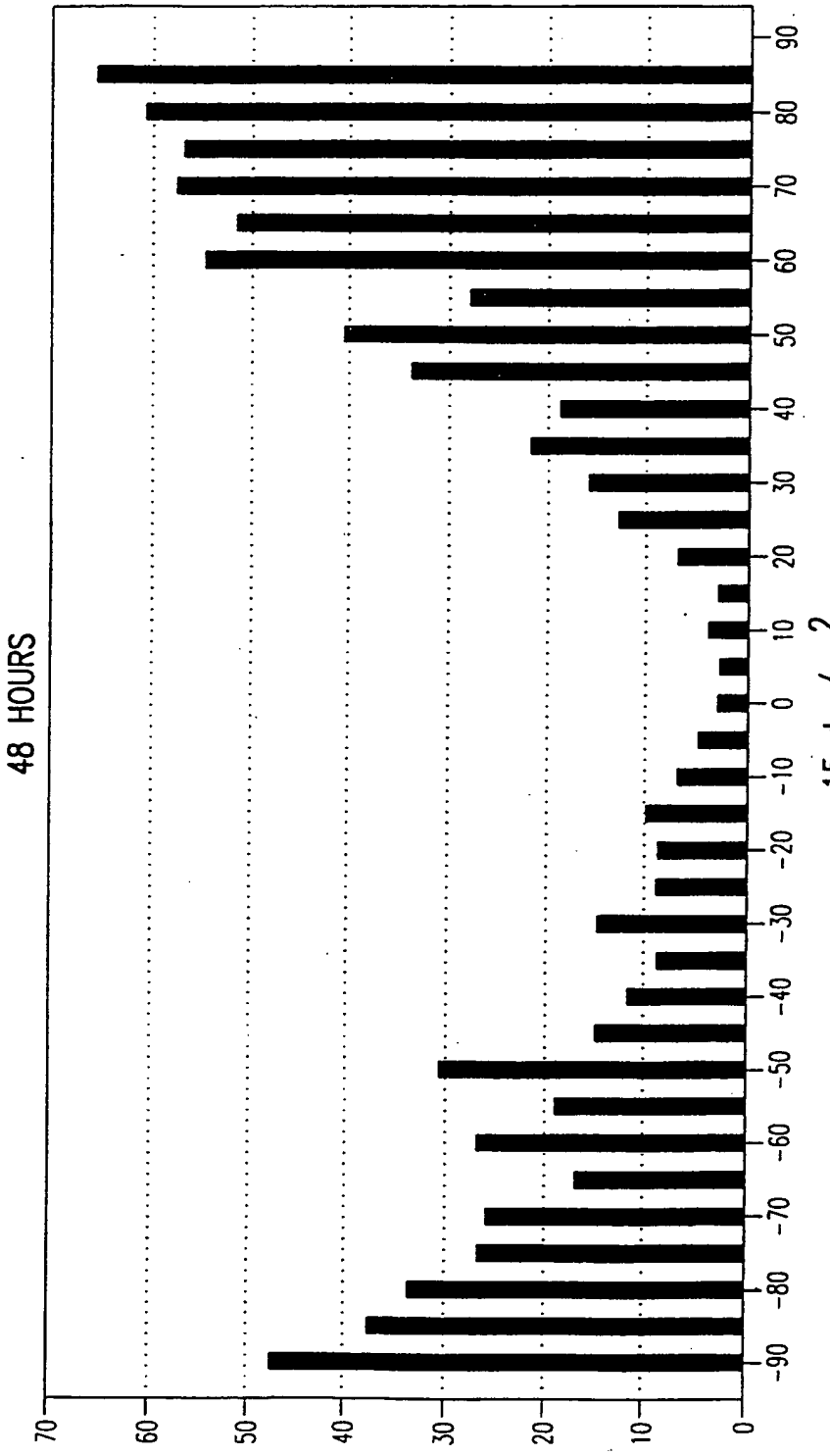

48 HOURS
15 dyn/cm2
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
70
60
50
40
30
20
10
0

FIG.3H

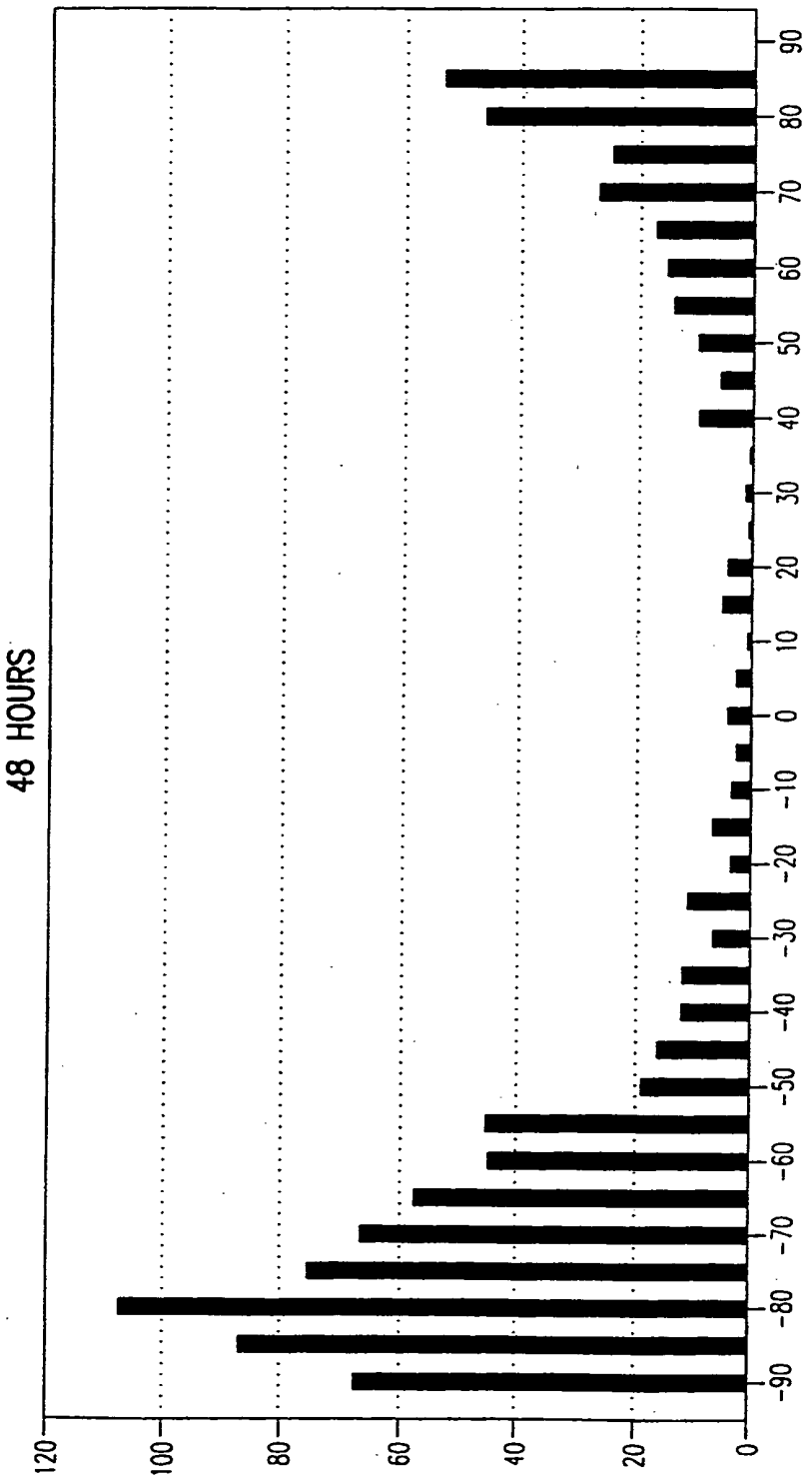

48 HOURS
120
100
80
60
40
20
0
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
20 dyn/cm2

FIG.31

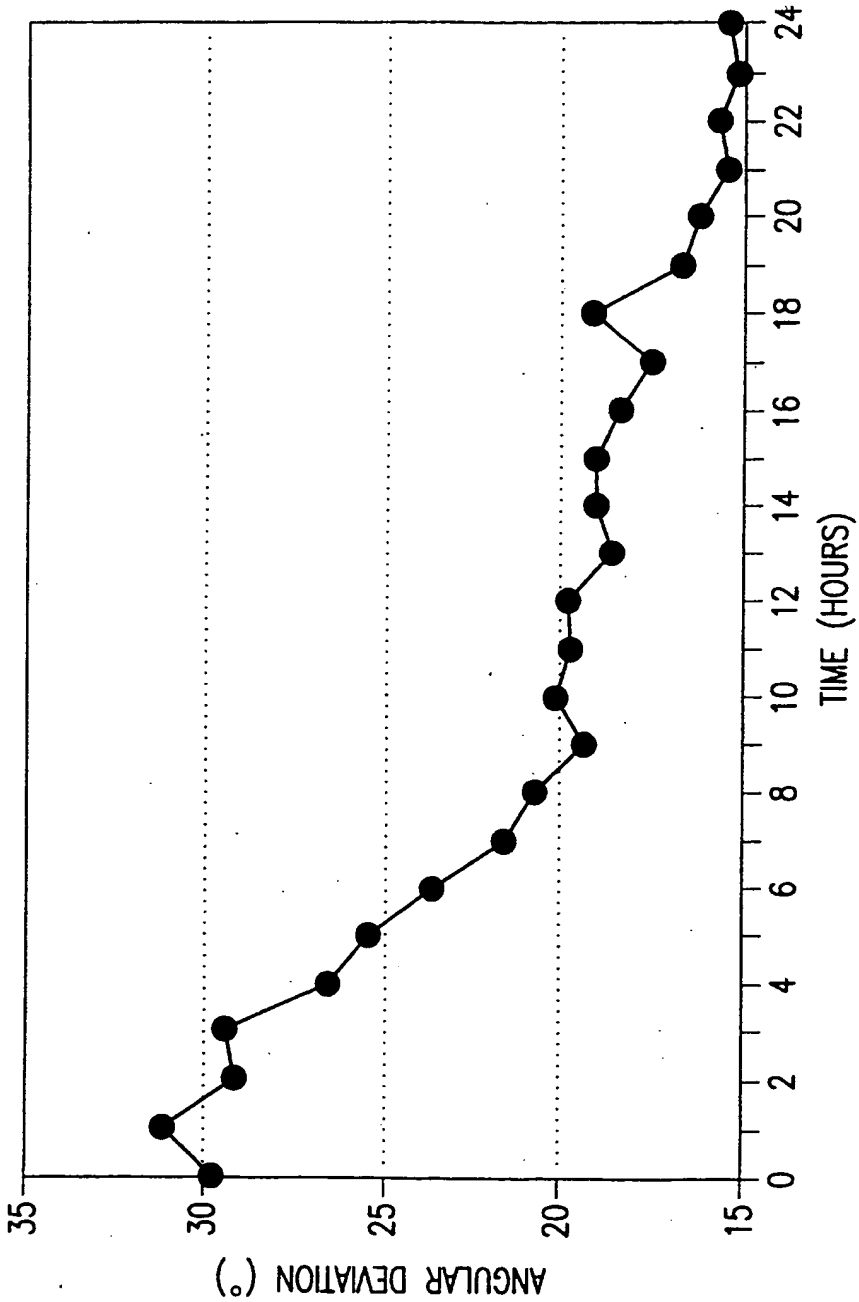

ANGULAR DEVIATION (°)
35
30
25
20
15
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (HOURS)

FIG.4

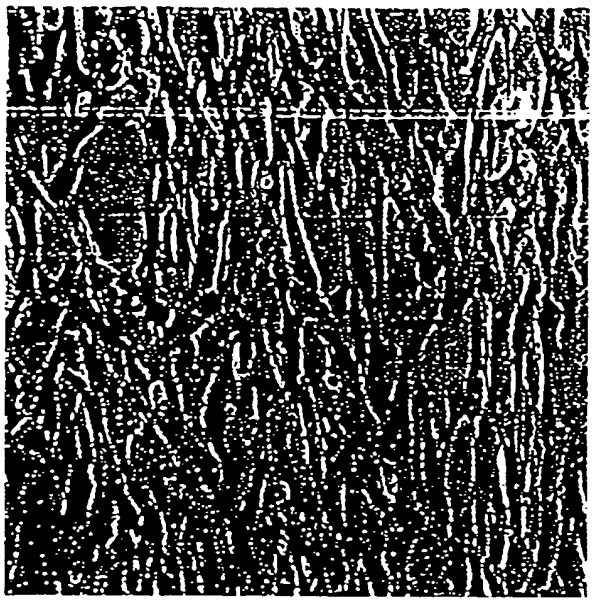


FIG.5A

FIG.5B

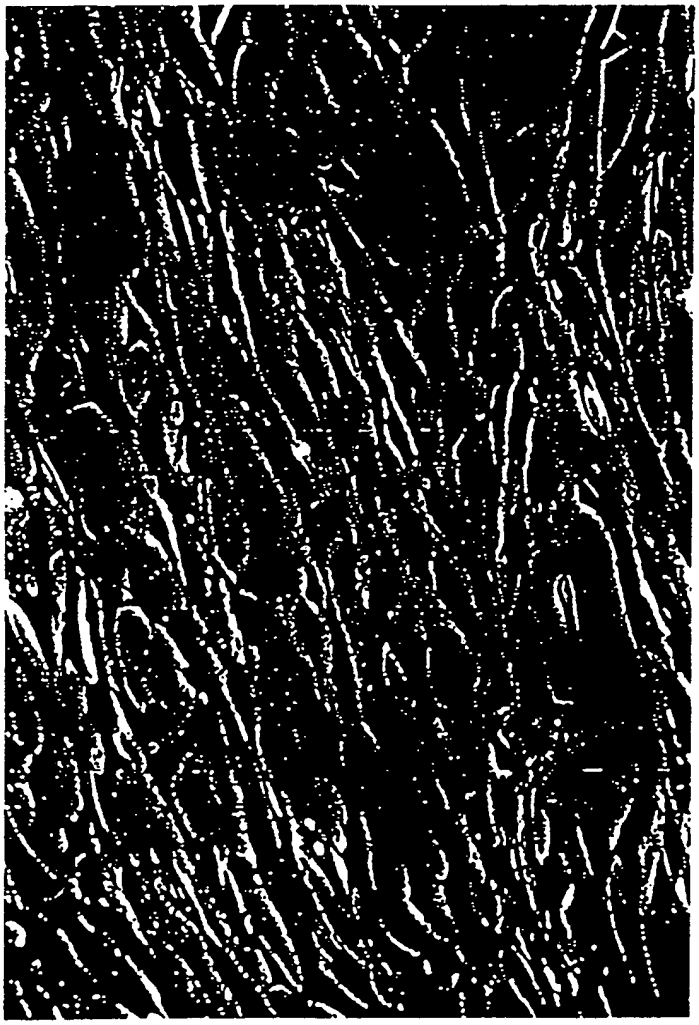


FIG.6A

FIG.6B

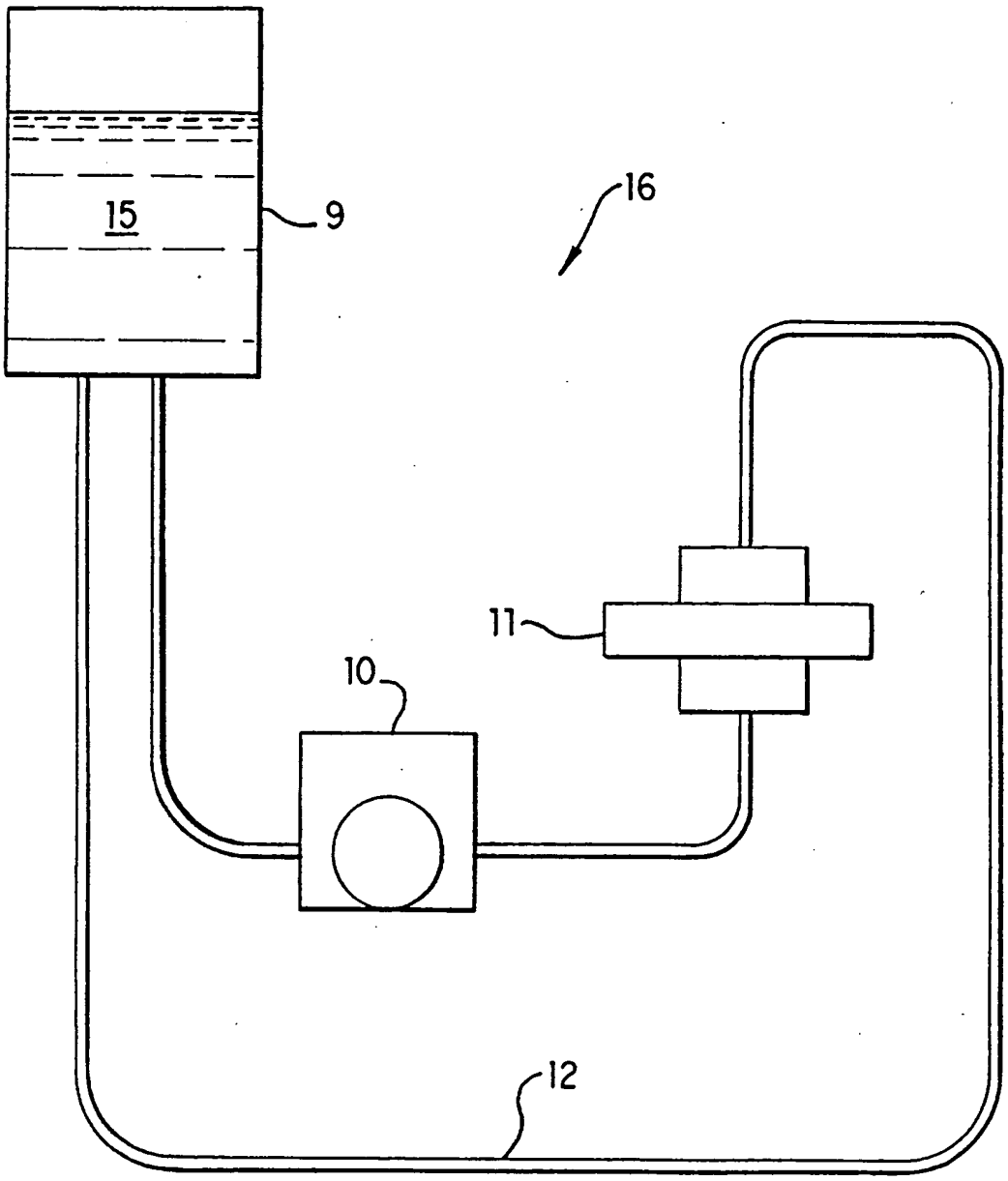


FIG.7

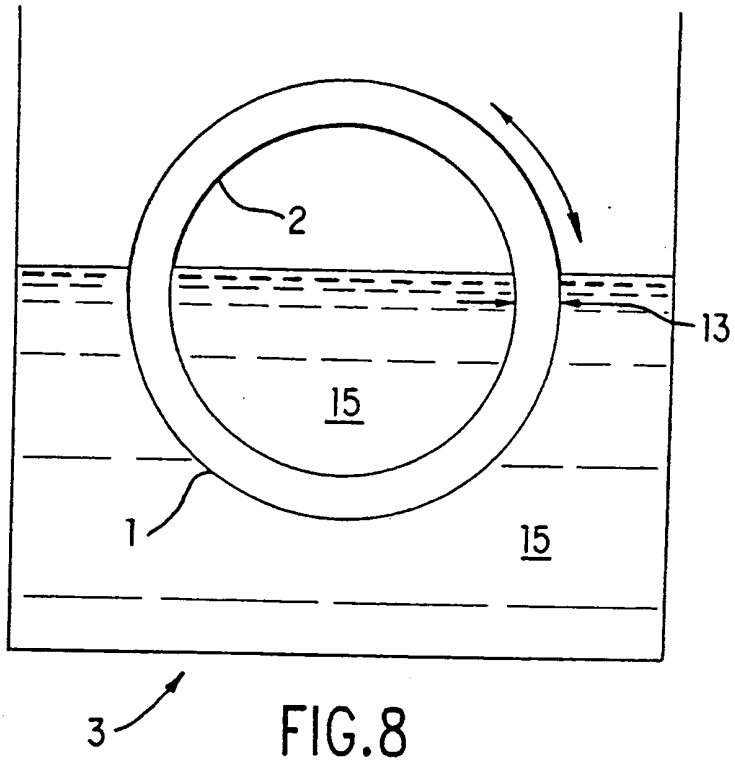


FIG.8

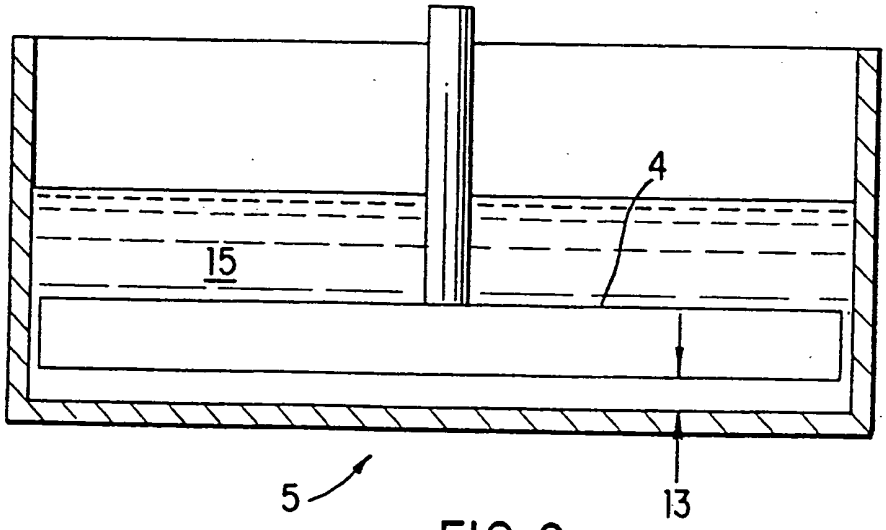


FIG.9

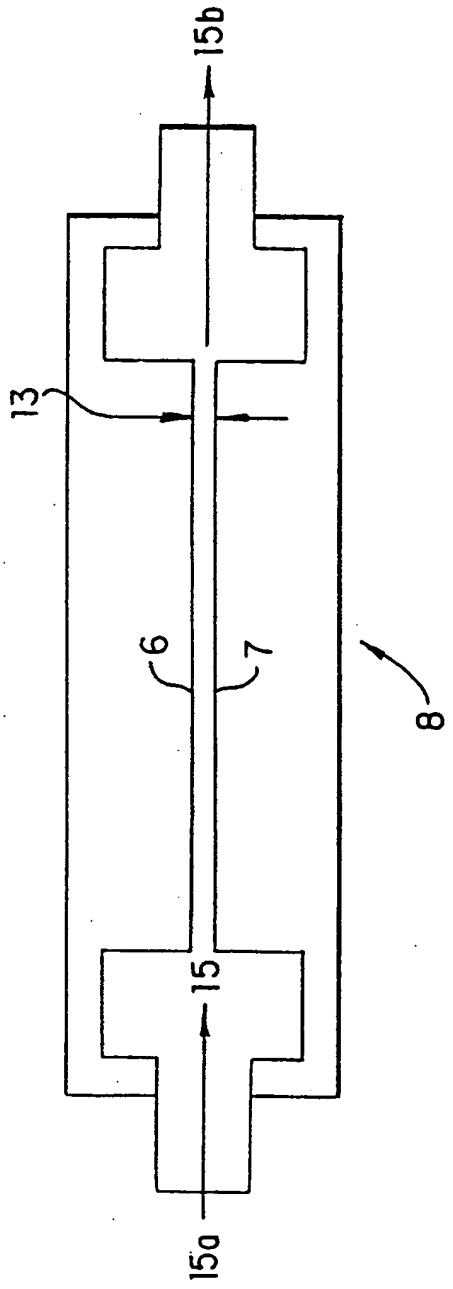


FIG.10

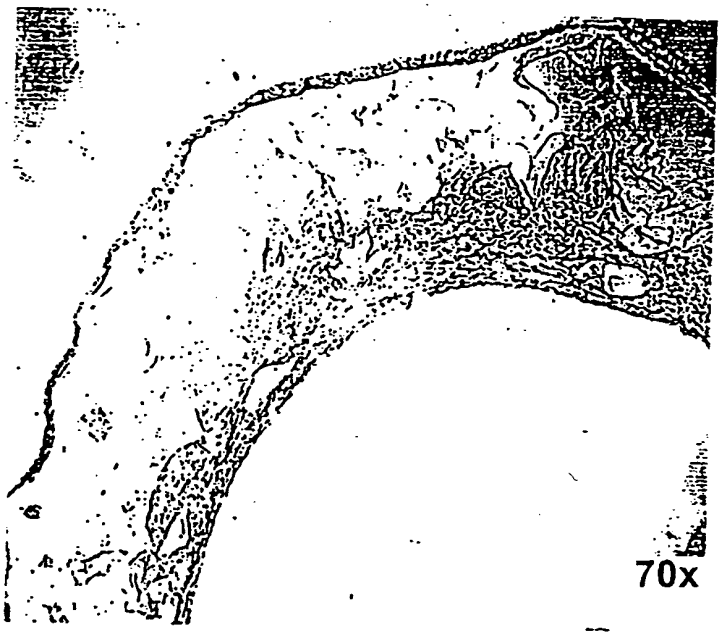


FIG.11A

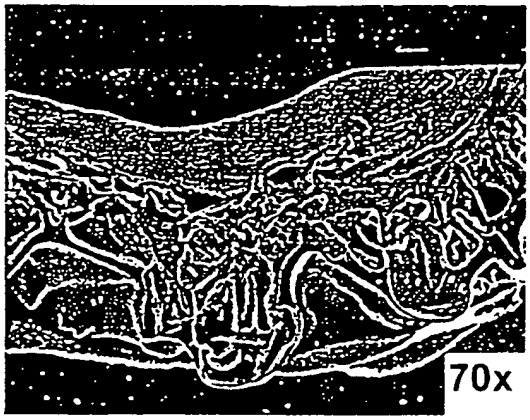


FIG.11B

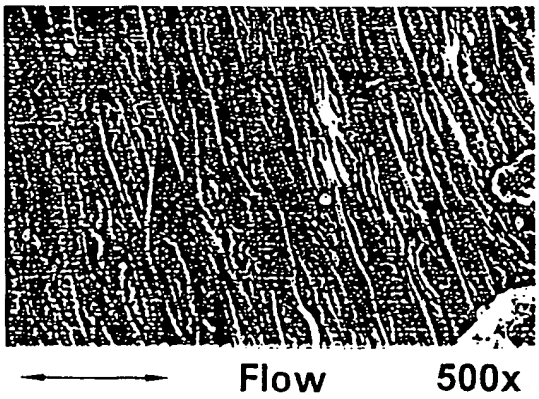
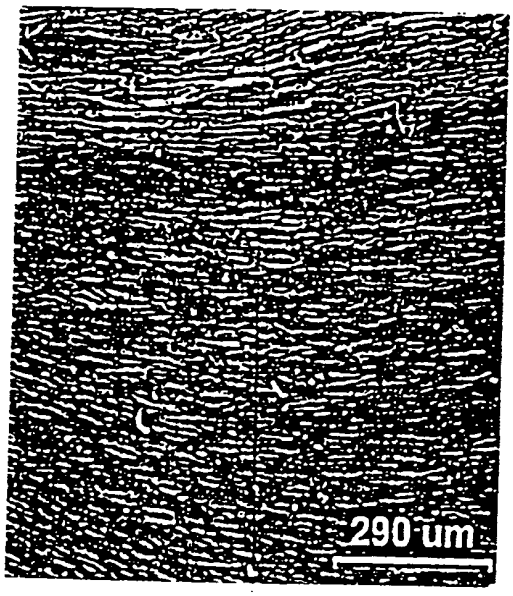


FIG.11C



100x

FIG.12A

500x

FIG.12B

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant: ADVANCE TISSUE SCIENCES, INC. [US/US]; 10933 North Torrey Pines Road, La Jolla, CA 92037-1005 (US).

(72) Inventors: LEE, Ann, A.; 680 Serra Street, Stanford, CA 94305 (US). ZELTINGER, Joan; 4136 Camino Ticino, San Diego, CA 92122 (US). KLADAKIS, Stephanie, M.; 1518 Lake View Lane, Atlanta, GA 30339 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW.

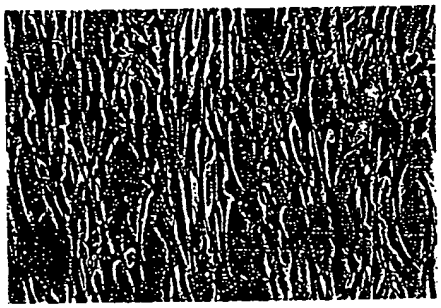
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent

[Continued on next page]

(54) Title: APPLICATION OF SHEAR FLOW STRESS TO SMOOTH MUSCLE CELLS FOR THE PRODUCTION OF IMPLANTABLE STRUCTURES

(57) Abstract: The present invention relates to methods for the growth of smooth muscle cells in culture for the production of tissue-engineered grafts or other implantable replacement structures. More specifically, the invention relates to the application of shear flow stress to smooth muscle cells in culture, wherein the cells align perpendicular to the direction of flow, thus more closely approximating the orientation of the cells *in vivo*. The resulting cultures and methods are useful for the production of improved vascular grafts, vessels and other implantable structures for the correction of defects or abnormal tissues in the body.

A



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WO 00/34442 A3

(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 99/29257

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/08 C12N5/06 C12N15/63 C12N5/22 C12N5/16
C12N11/16 C12M3/00 C12N11/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZIEGLER, T. ET AL.: "Co-culture of endothelial cells and smooth muscle cells in a flow environment: an improved culture model of the vascular wall?" CELLS AND MATERIALS, vol. 5, no. 2, 1995, pages 115-124, XP000864797 cited in the application abstract page 117, column 1, line 16 -page 119, column 1, line 24 page 124, column 2, line 33 - line 35	1-3,25
Y		21,29
Y	WO 95 25547 A (UNIVERSITY OF WASHINGTON) 28 September 1995 (1995-09-28) abstract page 3, line 27 -page 5, line 13 page 20; claim 1	21,29

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 July 2000	21/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuchs, U

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INTERNATIONAL SEARCH REPORT

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PCT/US 99/29257

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEE, A.A.: "Mechanisms of fluid shear stress-induced alignement in cultured vascular smooth muscle cells" FASEB JOURNAL, vol. 13, no. 5, 15 March 1999 (1999-03-15), page A1020 XP000929487 the whole document	1-3,8, 10,12,19
A	WO 97 49799 A (ADVANCED TISSUE SCIENCES) 31 December 1997 (1997-12-31) cited in the application the whole document	1-50
A	CUCINA, A. ET AL.: "Haemodynamic forces modulate simultaneously the release of growth factors and the organisation of cytoskeleton of aortic smooth muscle-cells" MINERVA CARDIOANGIOLOGICA, vol. 44, no. 12, December 1996 (1996-12), pages 637-643, XP000864786 the whole document	1-50
A	RESNICK, N. ET AL.: "Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 10, 15 May 1993 (1993-05-15), pages 4591-4595, XP002075319 the whole document	21-24, 27-30, 33-36

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ational Application No
PCT/US 99/29257

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9525547 A	28-09-1995	AU 2195095 A	09-10-1995
WO 9749799 A	31-12-1997	US 5792603 A	11-08-1998
		AU 3581797 A	14-01-1998
		CA 2261027 A	31-12-1997
		EP 0927245 A	07-07-1999